THE OESTROGENIC EFFECTS OF SUBTERRANEAN CLOVER (*TRIFOLIUM SUBTERRANEUM*)

UTERINE MAINTENANCE IN THE OVARIECTOMISED EWE ON CLOVER GRAZING.

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The oestrogenic effects induced in sheep grazed on dominant pastures of *T. subterraneum* var. Dwalganup have been described by Bennetts, Underwood and Shier (1946), Bennetts (1946 and 1947); the presence of an oestrogenic substance in extracts of the clover was confirmed by Robinson (1946 and 1949) and by Curnow, Robinson and Underwood (1948). Further studies of the effects induced in laboratory animals and sheep were reported by East, Underwood and Bennetts (1949). The purpose of this paper is to describe an experiment, the objects of which were to determine firstly whether the oestrogenic substance in clover would function like a natural oestrogen in preventing uterine atrophy in the castrate, and secondly to obtain, by this index, some further evidence on the seasonal variation in the potency of clover grazing.

EXPERIMENTAL DESIGN AND METHODS.

In April, 1947, an even line of 42 Merino ewes, aged approximately twelve months, were purchased from a non-clover property on which they had been bred. They were transferred to the Avondale Research Station, Beverley and maintained on grazing devoid of subterranean clover.

They were ear tagged and on May 15th and 16th, 40 were ovariectomised under chloral hydrate anaesthesia. The ewes were retained at Avondale until the 15th July, when the two entire ewes and two of the castrates were killed for examination. The following day the remaining 38 ovariectomised ewes were divided into two groups.

Group 1—"Non-clover grazing", retained at Avondale.

Group 2—"Clover grazing", transferred to a pasture dominated by subterranean clover on property T. This property, situated only 6 miles from Avondale, has been used throughout our investigations for experiments with sheep and for the supply of potent clover for experimental purposes. There was, moreover, a known history of severe infertility in the farm flock.

The two groups of sheep were maintained throughout the experiment under conditions of grazing and management which were as far as possible identical except for absence and dominance of subterranean clover in their respective pastures.

Two ewes from each group were slaughtered at intervals for examination until the conclusion of the experiment on 28/9/48 when the three ewes remaining in each group were killed. The intervals of slaughter selected were designed to give the most complete information concerning the correlation between the potency of the clover and uterine status. To this end examinations were made at monthly intervals during the green and early dry feed period, July to December, and less frequently during the dry late summer and autumn. In this connection experimental evidence, reviewed by Bennetts and Underwood (1949) had shown that the clover was potent throughout the green growing period with a rapid fall in potency at wilting time—mature dry clover has little potency.

The observations made at the time of slaughter comprised the following: clinical condition (notably evidence of mammary development), carcass weight, uterine measurement and weight, and histological examination of the genital tract. For this latter purpose portions of the uterus comprising material from each horn, at the same level, and of the body of the uterus were fixed in 10 per cent. formalin followed by secondary fixation in Vandergrift's (1942) solution. Paraffin sections were prepared and stained with haematoxylin and eosin for examination. In most instances a histological examination of the vagina was also carried out.

EXPERIMENTAL RESULTS.

The results of preliminary uterine examinations are given in Table 1.

TABLE 1.

Pretreatment examinations.

(Non-clover grazing at Avondale.)

Date	Ewe		Uterus		Uterus histological examination	Remarks
	No.	Status	Length cm.*	Weight gm.		
15/7/47	A 1	Castrate	11·0	10·0	Normal	Ovariectomised 15/5/47
	A 2	Castrate	7·5	5·6	Normal	15/5/47
	247	Entire	11·0	8·0	Normal	
	255	Entire	11·0	10·5		

* Internal os of cervix to junction of uterus and fallopian tube. The length of the cervix was also determined throughout the series but these data are not presented as they showed nothing of significance.

The results of examination of the uteri of the group 2 ewes which were transferred to dominant clover grazing on 16/7/47 when potent green clover was available, are tabulated in Table 2.

In group 1, maintained on non-clover grazing, atrophy of the uterus was marked within three months of ovariectomy, that is by 12/8/47, and thereafter the animals in this group gave a uniform picture, both in regard to uterine size and histology, throughout the experiment. This excludes three ewes which, on examination, were found to have ovarian fragments retained and two which were suspected of this although no ovarian tissue was found. The mean length of the uterus of 12 ewes was 10·5 cm. (range 8·0–13·0 cm.) and the mean weight 6·3 gm. (range 3·8–8·4 gm.). (In the other five, the mean length was 13·6 (11·0–16·0) cm. and the mean weight 14·4 (12·2–17·2) gm.)

TABLE 2.

Uterine examinations in group 2 ewes on clover grazing.

Date	Ewe No.	Uterus		Uterus histological examination	Remarks	Stages of growth of clover
		Length	Weight			
12/8/47	A 19	12·0 cm.	12·8 gm.	Normal	After one month on green clover	Green clover (potent)*
	A 39	15·0	14·7	Normal		
16/9/47	A 33	14·0	18·0	Normal	Slight udder development	Mature flowering green clover (potent)
	A 15	17·0	16·5	Normal	Do.	
16/10/47	A 17	15·0	13·3		Udder secretion	Early wilting stage (potency declining)
	A 21	12·0	10·8	Normal	Do.	
19/11/47	A 13	12·0	7·0	Atrophic. A few dilated glands		Dry clover (non-potent)
	A 31	10·0	7·7	Atrophic		
18/12/47	A 9	14·0	8·2	Atrophic		Dry clover (non-potent)
	A 27	16·0	14·8	Normal	Ovarian fragment present	
19/3/48	A 35	10·0	4·4	Atrophic		Dry clover (non-potent)
	A 29	10·5	5·5	Atrophic		
6/7/48	A 11	24·0	15·8	Normal		Green clover (potent) since early June
	No tag	14·5	9·3	Normal		
31/8/48	A 3	14·0	14·6	Normal	Some udder development	Maturing clover (potent)
	A 23	14·0	12·5	Normal		
28/9/48	A 5	13·0	11·1	Normal	Considerable udder development	Mature flowering clover (potent)
	A 7	14·0	17·0	Normal		
	No tag	18·5	18·4	Normal		

*"Potency" refers to results of biological assays carried out on numerous samples of clover at the various stages of growth; many of the samples assayed were obtained from the actual pasture on which the experimental sheep were grazed.

From Table 2 it can be seen that green clover grazing was adequate to prevent the atrophy of the uterus which normally results from ovariectomy; the normal structure of the uterus was maintained as evidenced by histological examination and the uterine weights were in all instances within the "normal" range. In that connection we have obtained the following uterine measurements for 10 healthy, entire, unbred anoestrous Merino ewes of corresponding age: Length 8·0–18·0 (11·2) cm.; weight 8·0–25·0 (11·6) gm.

This maintenance effect lasted from mid-July, 1947, when the sheep were first put on to clover, until mid-October, by which time the clover had wilted. Following this period until June 1948, during which no green clover was available, the uteri underwent atrophy and there was no significant difference between the clover and non-clover groups. From June onwards, green clover again became available and by early July uterine weight and histology had returned to their normal condition as in the former period of green grazing.

It appears that the duration of oestrogenic stimulation from the clover is confined to the growing period of the clover and does not persist into the November-December mating period. The increase in uterine weight and hypertrophy of the uterine glands are only temporary effects which are soon lost when the oestrogenic stimulus is removed. The histological appearance of the vagina, where examined, gave similar indications of the presence or absence of oestrogenic stimulus, the epithelium being commonly reduced to a single layer of undifferentiated cells in the absence of potent clover grazing. It was normal in appearance in group 2 ewes grazing on green clover. Body weight was, in general, better maintained on the non-clover grazing consisting principally of Capeweed (*Cryptostemma calendulaceum*), wild geranium (*Erodium botrys*), *Bromus spp.* and *Festuca spp.*, than on the subterranean clover pastures which contained practically no other herbage.

DISCUSSION.

It is well known that ovariectomy is followed by atrophy of all the component parts of the uterus and that this "castration atrophy" can be prevented, or the uterus restored almost to its normal state by adequate treatment with oestrogens. Published evidence on this aspect has been reviewed by Burrows (1949).

The main object of the experiment reported, was to determine whether the oestrogenic principle occurring in *Trifolium subterraneum* var. Dwalganup, in the amounts taken in by sheep grazing pastures dominated by this species, would in that respect function like a natural oestrogen.

The results obtained indicate that in sheep, as in other species, ovariectomy is followed by atrophy of the uterus and that this uterine atrophy can be prevented and the uterus restored to normal by the ingestion of the green clover, this effect being ascribed to an oestrogenic principle which has been demonstrated in the clover. It is unfortunate that the data presented are somewhat marred by the fact that in some instances, as reported, ovariectomy was incomplete. The results of the experiment are, however, quite clear cut.

In another experiment, not yet published, it was demonstrated that regular injections of stilboestrol were also effective in preventing uterine atrophy in the ovariectomised ewe.

Endeavours have been made to obtain quantitative estimations of the changes in oestrogenic potency of the clover at different stages of growth, using the extraction and assay method reported by Beck and Braden (1951). Although entirely satisfactory results have not been obtained, Braden (1950) has demonstrated that the clover is potent throughout the green growing period with a rapid fall in potency at wilting time and that mature dry clover has little potency.

Bennetts (1947) had reported squamous metaplasia in the secondary sex organs of the castrate male sheep maintained on green clover pasture with regression towards normality when the clover was dry. The results of these more recent observations with the ovariectomised female provide further confirmatory evidence of the variation in potency with stage of growth of the plant.

SUMMARY.

In the sheep as in other species of animals ovariectomy results in uterine atrophy.

This atrophy is prevented, or the atrophic uterus restored to its normal state by the ingestion of the oestrogenic principle of green *T. subterraneum* var. Dwalganup in amounts taken in by sheep grazing pastures dominated by this species. The "clover oestrogen" in this respect functions like a natural oestrogen.

The observations with ovariectomised ewes provide additional evidence that green clover is potent whereas dry clover has little potency.

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THE REMOVAL OF PROTEIN FROM THE SUBARACHNOID SPACE[1]

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Although the protein concentration in cerebrospinal fluid is normally very low, it may rise to quite high levels in a disease such as meningitis. The removal of extravascular plasma protein from other regions of the body such as the lungs and the pleural and peritoneal cavities is almost entirely by way of the lymphatics (Courtice and Simmonds, 1949a and b; Courtice and Steinbeck, 1950). Since the central nervous system and meninges are said to contain no lymphatics, whether the rate and manner of removal of plasma proteins from the subarachnoid space are affected by this anatomical feature seemed worthy of investigation.

METHODS.

The blue dye, T1824, was used to label the plasma protein. It has been shown that this dye is quite firmly bound to protein *in vitro* (Rawson, 1943; Allen and Orahovats, 1950) and *in vivo* (Gregersen and Rawson, 1943; Courtice and Simmonds, 1949). Injections were made into the cerebrospinal fluid of the cisterna magna or of the lumbar sac, and in all cases homologous plasma or the animal's own plasma was used. In rabbits 0·5 ml./kg. body weight of heparinized plasma containing 2 mg. dye/ml. was injected, while the dosage in cats was 1 ml./kg. body weight containing 2 or 4 mg. dye/ml. In some instances, however, the volume of injected plasma was varied. Injection pressures were controlled either by slowly introducing the fluid at a measured pressure of less than 180 mm. H_2O or by first withdrawing a volume of C.S.F. equal to the volume of plasma to be injected. Control experiments in cats, using a 1 mm. bore manometer, showed that cerebrospinal fluid pressures might rise to 200 mm. C.S.F. immediately after a replacement injection of 1 ml./kg. but returned practically to normal values within a few minutes.

Dye plasma was introduced into the cisterna magna by passing a fine, short-bevelled needle through a nick in the skin, a small 3-way tap being used for the replacement injection. For lumbar subarachnoid injections a cannula was tied into the lumbar sac distal to the exit of the roots of the lumbo-sacral plexus. In other control experiments injections were made not into the C.S.F. but into the tissues around the cord by passing a fine, angled needle through the ligamentous roof of the vertebral canal at the lumbo-sacral junction. This needle had a closed, round tip with a hole about 2 mm. from the end.

Dye concentrations in plasma and C.S.F. were determined with a Spekker photoelectric absorptiometer after extraction of the dye with *n*-butyl alcohol (Harington, Pochin and Squire, 1940). Blood samples for plasma were taken from the ear vein in rabbits and from a femoral arterial cannula in cats; dry heparin was used as an anti-coagulant. For dye deter-

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minations the C.S.F. was diluted with undyed plasma. Total nitrogen and N.P.N. concentrations in C.S.F. and plasma were determined by micro-Kjeldahl digestion and direct Nesslerization using a Spekker photoelectric absorptiometer, N.P.N. being determined after precipitation of protein with trichloracetic acid.

RESULTS.

Injection by the cisterna magna route.

Rate of absorption. The rate of removal of dye-plasma from the subarachnoid space was investigated in rabbits. The animals were anaesthetized with a small dose of nembutal intravenously, supplemented by open ether. Complete recovery from anaesthesia took one hour or more and the animals then remained in their cage for the rest of the experiment. Blood samples were taken at intervals, and at the end of the experiment, which lasted for one to 24 hours, the animal was again anaesthetized, a sample of C.S.F. was withdrawn from the cisterna magna and autopsy performed after killing the rabbit by bleeding out under anaesthesia.

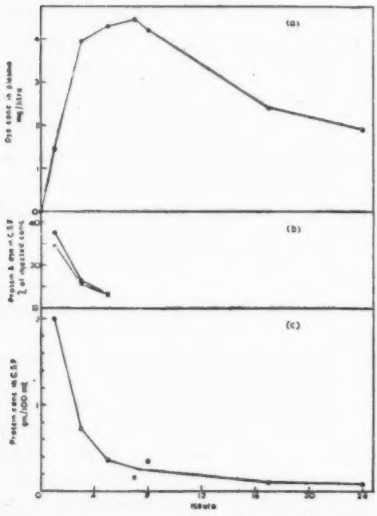

Fig. 1. Absorption of dye-protein from the subarachnoid space after cisternal replacement of 0·5 ml, C.S.F./kg. body weight by an equal volume of rabbits' heparinized plasma containing 2 mg. dye/ml. (a) Average dye concentrations, in mg./l., in the circulating plasma. (b) Average dye concentrations and protein concentrations in C.S.F., expressed as percentages of dye and protein concentrations in injected plasma. ■, protein; x, dye. (c) Average protein concentrations in gm./100 ml. C.S.F.

The results of these experiments are summarized in Fig. 1. Fig. 1 (a) shows that dye-protein passes quite rapidly from the subarachnoid space into circulation in the first 3 to 5 hours and thereafter much more slowly. After 8 hours plasma dye concentrations decline, presumably because the rate of disappearance of dye-protein from the circulation exceeds the rate of entry of dye-protein from the subarachnoid space. The peak values observed for plasma dye concentration correspond to an absorption of at least 20 p.c. of the injected dye-protein within 3 to 5 hours, since the plasma volume in the rabbit is approximately 40 ml./kg. body weight. The plasma dye concentration curves in individual rabbits agreed well with the curve in Fig. 1 (a) in which average values are plotted, but in some rabbits no dye absorption was apparent until after the first hour. Peak values for plasma dye concentration varied from 3·0 to 5·7 mg./litre in different rabbits.

From Fig. 1 (c) it is evident that the protein concentration in the cerebrospinal fluid withdrawn falls rapidly in the first 5 hours, corresponding to the period of rapid absorption into the circulation, while after 17 hours little protein remains. In the 4 rabbits killed after 24 hours the protein concentrations were 0·16, 0·08, 0·07 and 0·06 gm./100 ml. C.S.F. Two factors contribute to the decline in protein concentration—mixing of the injected dye-plasma with C.S.F., especially in the first hour or so, and absorption of the dye-protein.

Fig. 1 (b) suggests that the dye and protein disappeared from the C.S.F. at the same rate during the first 5 hours. After this period dye and protein concentrations were too low to permit accurate estimation of both in the sample of C.S.F. withdrawn, so only protein estimations were made. It could be seen, however, that the C.S.F. became progressively less blue and at 24 hours was no more deeply dyed than the plasma. When dyed human plasma was injected into the cisterna magna in 3 rabbits the dye concentrations and anti-human precipitin titres in the circulating plasma increased together. Dye and protein remain in combination during absorption, therefore, since they disappear together from the C.S.F. and appear together in the blood stream.

Routes of absorption. Autopsies were performed on all these animals to study the distribution of the injected dye-plasma and the lymphatic pathways of absorption. It was found that mixing occurred more slowly with the C.S.F. in the spinal cord than with that in the cerebral subarachnoid space. In rabbits killed at 1, 3 and 5 hours the C.S.F. in the lumbar sac was less blue while from 17 hours onwards it was more blue than that in the cerebral region. In contrast to this slow mixing in the spinal C.S.F., dye was seen throughout the whole of the cerebral subarachnoid space, in its extensions in the region of the cribriform plate and along the optic nerve, and often in the retrobulbar tissues in animals killed as early as 1 hour after injection. Regarding the lymphatic pathways of absorption, in all cases the cervical lymph ducts and the lymph glands at the angle of the jaw were stained blue, showing that an appreciable amount of dye-protein was being absorbed into these lymphatics through the cribriform plate. In the spinal cord the epidural tissue in the region of the posterior root ganglia in the lumbar, sacral and cervical regions frequently showed some dye staining, although this was slight. In no case, however, was there any visible dye in the lymph glands into which drained the small lymph vessels emerging from the vertebral canal; nor were these lymph vessels blue. This indicates that although some dye-protein may escape along the spinal nerve roots the quantity is too small to make the draining lymphatics visibly blue.

TABLE 1.

The absorption of dye-protein into the lymph and directly into the bloodstream in cats, after replacement of C.S.F., approximately 1 ml./kg. body weight, by an equal volume of the animal's own heparinized plasma containing dye, 4 mg./ml., injected into the cisterna magna. Dye in lymph and circulation is expressed as a percentage of the dose of dye injected. Dye in circulation is calculated from plasma dye concentration at end of experiment × plasma volume (35 ml./kg. body weight).

Dye injected (mg.)	Duration of experiment (hours)	Percentage of dose collected in cervical lymph	Percentage of dose collected in T.D. lymph	Percentage of dose in circulation
8·0	3¾	3·3	—	10·8
12·0	4½	4·8	0·43	14·9
11·6	3½	6·6	0·48	12·1
12·0	3½	4·2	—	18·6

Percentage of dose in cervical lymph calculated as twice the percentage collected from left cervical duct except in the first experiment listed where lymph was collected from both right and left ducts, the dye collected being 1·6 p.c. and 1·7 p.c. of injected dose, respectively.

To provide a quantitative comparison of the amounts of dye-protein absorbed into the different lymphatics as well as directly into the circulation, cats anaesthetized with nembutal were used. Lymph from the cannulated cervical and thoracic ducts was collected for periods of 3½ to 4½ hours after the replacement injection into the cisterna magna of 1 ml./kg. of

the animal's own dyed plasma containing 4 mg./ml. dye. Table 1 shows that the total dye collected in the lymph was much less than that which could be calculated to have passed directly into the circulation, while the amount of dye recovered from the thoracic duct lymph is very small compared with that in cervical lymph.

Although in control experiments pressure changes are not great after ordinary replacement injection of 1 ml. C.S.F./kg. body weight, the absorption of dye-plasma was also studied after smaller replacement injections at a measured pressure in 3 cats. In these animals all the main lymphatics were tied off in the neck and 0·5 ml. C.S.F./kg. body weight was replaced by the same volume of their own heparinized plasma containing 2 mg. dye/ml. at an injection pressure which was not allowed to exceed 180 mm. water. On an average, 8·6 p.c. of the injected dye-protein was present in the blood plasma at 5 hours despite lymphatic ligation. The passage of dye-protein directly into the circulation cannot be ascribed, therefore, to the use of grossly abnormal injection pressures.

Demonstration of a difference between the plasma dye concentration in the sagittal sinus and in the carotid artery would provide additional evidence of protein absorption directly into the blood vessels. In six out of eight paired samples from two cats the dye concentration in the sagittal sinus plasma was 1·1 to 2·0 mg./litre greater than that in the femoral arterial plasma. In another experiment no significant differences were detected. The results are suggestive but it is felt that for more conclusive results a larger experimental animal would be preferable.

Injection by the lumbar route.

The somewhat slow movement of the dyed plasma through the spinal C.S.F. after injection into the cisterna magna might have been responsible for the much greater lymphatic absorption from the cerebral subarachnoid space. It was, therefore, decided that injection by the lumbar route should be performed, so that there would be from the beginning a high concentration of dyed plasma in the spinal C.S.F.

TABLE 2.

Dye absorption into thoracic duct lymph and directly into bloodstream after lumbar injection in cats of their own heparinized plasma containing dye, 2 mg./ml.

Dose of dye (mg.)	Duration of experiment (hours)	Final dye concentration in T.D. lymph (mg./l.)	Final dye concentration in plasma (mg./l.)	Percentage of injected dye recovered in T.D. lymph	Percentage of injected dye in circulation
		(a) Injection into subarachnoid space			
2·8	4	trace	2·9	trace	9·8
6·0	5½	3·3	8·0	0·72	15·9
		(b) Injection into extradural tissues			
3·0	3	7·8	3·1	3·87	10·9
2·7	3	6·2	1·4	1·57	4·9
3·2	3½	15·3	5·9	6·69	20·6

In one group of experiments on cats, dyed plasma was introduced through a cannula tied into the lumbar sac, either slowly under a measured pressure not exceeding 300 mm. water or more rapidly by syringe without measuring the pressure. No lymphatics were cannulated or tied in these experiments, but the lymphatic pathways of absorption were determined qualitatively at autopsy 7 to 8 hours after injection. In all cases the deep cervical lymph nodes

and ducts were stained with dye as in the experiments after cisternal injection, while the blood also contained dye. In the spinal region, however, the lymph vessels and nodes draining the vertebral canal were not visibly blue although in some cases the extradural tissues in the region of the posterior root ganglia were slightly stained.

In two other experiments in cats the thoracic duct lymph was collected. Table 2 (a) shows that the amount of dye in this lymph was not greater than in the experiments with cisternal injection.

It seems, therefore, that in these animals dye-protein escapes from the spinal subarachnoid space in but insignificant amounts. The small amount of dye which does appear in the thoracic duct lymph probably comes from the blood after absorption directly into the blood-stream, since in cats 4 to 5 p.c. of a dose of dye injected intravenously was recovered in the thoracic duct lymph in 5 hours, indicating the extent of the normal leakage of protein from the visceral circulation. In the experiments in Tables 1 and 2, the amount of dye recovered in the thoracic duct was always less than 5 p.c. of that present in the circulating plasma.

It seemed important, however, to see if a prolonged high pressure within the subarachnoid space would accelerate the passage of dye-protein through the meninges into the extradural tissues. The results in 5 cats, summarized in Table 3, do not suggest that such pressures as might occur in meningitis or spinal movement would increase the passage of protein through the meninges into the extradural tissues, where it might be readily absorbed. The extradural leak recorded as "slight" consisted of light blue staining of the posterior spinal ligament and epidural fat pads, especially over the posterior nerve roots. There was no visible dye in the vertebral lymphatics. The extradural leak recorded as "very slight" consisted of light blue staining on the dural aspect of a few epidural fat pads over the lumbar nerve roots.

TABLE 3.

Prolonged injection at maintained pressure into lumbar subarachnoid of cats of the animal's own heparinized plasma, containing 2 mg. dye/ml.

Duration of injection (hours)	Burette pressure (mm. water)	Range of cisternal pressure (mm. C.S.F.)	Total dye-plasma injected (ml./kg.)	Plasma dye conc'n at end of injection (mg./l.)	Extradural leak
2	varied 0–200	varied with burette pressure	0·75	1·9	none
5	300	170–260	1·0	2·2	slight
3½	450	320–380	1·6	19·5	slight
5	450	300–430	2·6	12·5	v. slight
5	150	120–160	1·0	3·2	v. slight

Although there was practically no spinal absorption when all the dye plasma was injected under the dura-arachnoid even when the C.S.F. pressure was maintained at a high level for several hours, accidental leakage into the tissues around the cord always resulted in pronounced staining of the lymphatics issuing from the vertebral canal. In some experiments, therefore, the whole dose was purposely injected into the extradural tissues around the cord in the lumbar region. Both in cats and in rabbits this dye-plasma rapidly entered the lymphatics, so that at autopsy the lymph vessels emerging from the vertebral canal and the lymph nodes into which they drained soon became stained a deep blue. The amount of dye-protein passing into the lymphatics is shown in Table 2 (b). It can be seen that an appreciable proportion of the absorbed dye-protein passed into the thoracic duct lymph. In 3 to 3½ hours an average of 4 p.c. of the injected dose was recovered in the lymph and the dye concentration in the thoracic

duct lymph was much greater than that in the blood. Passive movement of the spinal column greatly increased the lymphatic removal of the dye. This is shown in an experiment depicted in Fig. 2. Abdominal massage in the absence of spinal movement, however, did not increase the lymphatic removal of dye-protein very much, although the thoracic lymph flow was temporarily increased. Hence the six-fold increase with passive flexion and extension of the spine cannot have been due to compression of the abdomen.

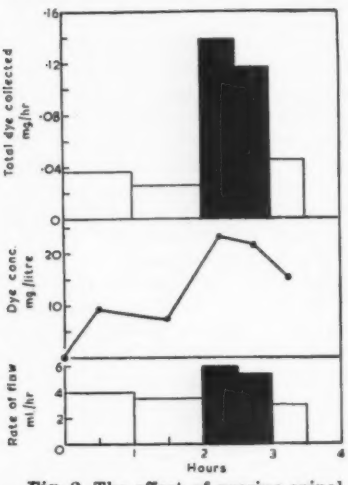

Fig. 2. The effect of passive spinal flexion and extension on the lymphatic removal of dye-protein after the injection into the lumbar extradural tissues of a cat of 0·5 ml./kg. of the animal's own plasma containing 2 mg. dye/ml. The graphs represent the lymph flow and the concentration, and amount of dye in the lymph collected from the cannulated thoracic duct. Black areas, passive movement of the spine.

It appears therefore that protein passes out of the C.S.F. along the nerve roots with difficulty, but that once it becomes extradural it is freely taken up by the lymphatics. The onward passage of this protein is then accelerated by movement of the spinal column. In all these experiments where dye-plasma was injected extradurally, the C.S.F. withdrawn from the cisterna magna was always colourless, which suggests that extradural protein probably is removed by the lymphatics and does not pass back through the meninges into the C.S.F. even though the injection pressure is high.

DISCUSSION.

The rate of absorption. The amount of injected protein removed from the C.S.F. can only be assessed indirectly since the total volume of C.S.F. cannot be accurately measured and mixing is not uniform. The fall in the concentrations of dye and of protein in the C.S.F., the rise in the dye concentration of the circulating plasma and the distribution of the dye at autopsy, when considered together, give a reasonably accurate account of the progress of protein absorption from the subarachnoid space. Thus 24 hours after injection in rabbits there was little dye or protein in the cisternal sample, very little to be seen in the subarachnoid space at autopsy and plasma dye concentrations had fallen far below peak values. Hence, nearly all the dye-protein must have been absorbed. In the earlier stages of absorption study of the C.S.F. alone would give ambiguous results. Mixing of the injected dye plasma was frequently irregular. At five hours the fluid in the lumbar sac was often only lightly dyed and there was evidence of uneven mixing in the cerebral subarachnoid space. However, the rapidly rising dye concentrations in the circulating plasma and the early staining of the lymph nodes show that there was a fairly rapid absorption of dye-protein as well as dilution of the injected dye plasma by undyed, protein-free C.S.F.

One of the difficulties encountered in these experiments was leakage at the injection site on withdrawal of the needle. The dye-labelling of the injected plasma revealed post mortem the degree of leakage from the cisterna into the neck muscles. Any leakage would lead to an overestimate of the amount of dye-protein absorbed, assessed by the concentration of dye-protein in the fluid withdrawn later by cisternal puncture. On the other hand, it would lead to an underestimate of the rate of absorption determined from dye concentrations in the circulating plasma, since control experiments showed that absorption from the neck muscles was very slow. Experiments were discarded where gross leakage was apparent at autopsy. Where a small amount of leakage was found at autopsy the results were included in the series, for it was found that plasma dye concentration curves and protein concentrations in C.S.F. did not differ materially in these rabbits from those in which no leakage was detectable.

Pathways of absorption. With regard to the lymphatic pathways, it is generally agreed that there are no lymphatics within the central nervous system or meninges. Protein, to reach the collecting lymphatics, has to pass through the meninges. Anatomical investigations have shown that solutes and particles pass out most readily in certain places. The most important cerebral route for escape into the tissue spaces and lymphatics appears to be along the extensions of the subarachnoid space around the olfactory filaments. Thus the material reaches the rich lymphatic plexus in the submucosa of the nasopharynx (Yoffey and Drinker, 1939). Carbon particles have also been shown to pass through the arachnoid sleeves around the optic nerves into the retrobulbar tissues (Field and Brierley, 1949) and dye-protein was found to behave similarly. Particles pass most readily out of the spinal subarachnoid space from the cul-de-sac just proximal to the posterior root ganglion of each spinal nerve, especially in the region of the second and third cervical nerves and of the three large roots contributing to the formation of the sciatic nerve (Brierley, 1950). Dye-protein might reach the deep cervical lymph, after cisternal injection, by olfactory, optic or cervical pathways of escape. However, prominent dyed lymphatics could be traced up to the nasopharynx, which was deep blue, while dyed lymphatic connections with the cervical extradural tissues could not be demonstrated. It is probable, therefore, that most of the dye-protein escaped by the olfactory route.

The results of experiment show quite clearly that the spinal lymphatic absorption of protein into thoracic duct lymph is small compared with the absorption by nasopharyngeal lymphatics into the cervical ducts (Tables 1 and 2). This is not unexpected, since the volume of C.S.F. in the spinal subarachnoid space is small and the rate of mixing of spinal with cerebral fluid is slow. Even under the most favourable conditions, when dye-plasma is injected directly into the lumbar sac and injection continued for long periods at moderately high but not excessive pressures, the passage of dye-protein through the spinal

meninges and into the lymphatics seems slow. It is likely that the difference between cranial and spinal absorption resides mainly in the meningeal barrier, since dye-protein injected into the lumbar extradural tissues passes quite rapidly into the lymphatics (Table 2).

In other regions where the removal of extravascular protein has been studied the lymphatics have been found to remove most, if not all, of the protein. This does not appear to be true for protein exuded into the C.S.F. Tying off all the major lymphatic pathways in the case of the pleural and of the peritoneal cavity virtually abolishes absorption of dye-protein. In the case of the subarachnoid space, considerable amounts of protein entered the circulation although the main lymph trunks were tied off. It seems unlikely that minute lymph channels or subsidiary lymphatico-venous entries of the type described by Job (1918) could account for the passage into the circulation of two to three times the amount of dye passing along the main channels. Since Weed (1914) showed that ferrocyanide was absorbed from the C.S.F. mainly through the arachnoid villi into the dural venous sinuses, it has been generally accepted that this constitutes the main pathway for absorption of the fluids and solutes of the C.S.F. It is reasonable to suggest, therefore, that the protein which is absorbed directly into the circulation passes in through the arachnoid villi.

Possibly, injection of plasma into the subarachnoid space may upset permeability at the absorbing surface. Flexner and Amoss (1914) showed that horse serum injected intraspinally in monkeys produced an aseptic meningitis while homologous serum gave similar but less pronounced changes (Flexner and Amoss, 1917). The fresh, heparinized homologous dye-plasma used in the present experiments did not, apparently, interfere with permeability to this extent. In the experiments with rabbits, the dye and protein concentrations in the C.S.F. fell at the same rate as absorption proceeded, whereas dye concentrations should have fallen proportionately more than protein concentrations if dye-protein were being absorbed and undyed protein passing out into the C.S.F.

SUMMARY.

The absorption of protein from the subarachnoid space of cats and rabbits has been studied by injecting under controlled pressure homologous heparinized plasma labelled with the blue dye, T1824, and determining the concentrations of dye and protein in C.S.F., circulating blood plasma and lymph.

In rabbits, initially anaesthetized only, 0·5 ml./kg. body weight was absorbed within 24 hours.

In anaesthetized cats, the amount of dye-protein passing directly into the circulation was two to three times the amount recovered from the cervical lymph during an experimental period of 3 to 4 hours, and the amount recovered from the thoracic duct lymph was negligible. This was true for injection of dye-protein into the lumbar subarachnoid space as well as for cisternal injection.

Post mortem examination showed, however, that a little dye-protein passed out in the region of the posterior root ganglia, but this was insufficient to stain the lymphatics and lymph nodes draining the vertebral canal even when a prolonged high C.S.F. pressure was maintained.

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THE HAEMAGGLUTININ OF HAEMOPHILUS PERTUSSIS

III. EXTRACTION OF THE ANTIGEN FROM THE BACTERIA AND ITS STABILIZATION BY ADSORPTION.

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Previous reports have dealt with the production and properties of haemagglutinin, its rôle as a virulence factor and immunizing antigen in mice and the antigenic structure of the haemagglutinating complex (Keogh, North and Warburton, 1947; Keogh and North, 1948; Fisher, 1948 and 1950). In the present paper the extraction of the haemagglutinin from the organisms, its adsorption to, and elution from, aluminium phosphate floccules, and the properties of the adsorbed product will be described.

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MATERIALS AND METHODS.

Method of preparation of adsorbed haemagglutinin.

For the experiments reported in this paper the adsorbed haemagglutinin was prepared from the virulent strain P61, but other strains have been used satisfactorily. The cultures were maintained in the lyophilized state and were revived not more than a fortnight prior to inoculating each batch of fluid medium; the number of intermediate sub-cultures was kept as low as possible. Cohen and Wheeler's (1946) medium, developed from Hornibrook's (1939) original formula, and modified by the addition of 1 p.c. soluble starch and excess yeast dialysate, was dispensed in lots of 150 ml. in Roux bottles, and each lot was inoculated with a loopful of a twenty-four hour culture grown on Bordet-Gengou or other suitable solid medium.

The Roux bottles were placed flat in the incubator at 36·5° C. and not disturbed for three or four days. (The actual time of incubation for maximum growth compatible with good haemagglutinin production varied slightly with different strains, but did not exceed four days.) The bottles were then shaken gently to suspend the culture, and the organisms spun down hard in sterile centrifuge tubes. The supernatant was removed and, since it contained haemagglutinin, was put aside for use in serum titrations.

The method employed for elution of haemagglutinin is based on unpublished observations of Keogh, which showed that the solubility of haemagglutinin in extracting fluid is at a minimum at pH 4·6, and is favoured by increasing or decreasing the hydrogen ion concentration. Extracted haemagglutinin is, however, rapidly inactivated in solutions more acid than pH 4·6, or more alkaline than pH 10. Elution of haemagglutinin is favoured by increasing the salt concentration of the extracting fluid. Keogh found that elution from the bacterial bodies was complete after 15 minutes at room temperature in 1 M aqueous solution of sodium chloride at pH 8·5, and that haemagglutinin could be precipitated from such solution by addition of enough distilled water to reduce the sodium chloride concentration to 0·15 p.c. or by half saturation with ammonium sulphate (Keogh, 1948).

The adsorbed antigen was prepared by the following method. The sedimented bacteria were washed once in sterile 0·2 p.c. NaCl at pH 5·5. The washings were discarded and the extracting medium consisting of sterile 1 M NaCl buffered with 0·01 M Na_3PO_4 added, using 100 ml. for each 150 ml. of culture. A high speed stirrer (5,000 r.p.m.) was used for a few seconds to disperse the organisms and to accelerate the extraction. The resultant somewhat viscous mixture which had a pH of 9·5–10·0, was allowed to stand for two to three minutes and then spun hard for a half hour. The cloudy supernatant was retained. The organisms were again extracted with a further lot of the same medium equal in volume to one quarter of that used for the first extraction, and centrifuged. The bacterial debris was discarded, the extracts pooled and titrated for haemagglutinin content. An equal volume of $AlPO_4$ suspension, prepared by the method of L. B. Holt (1950) and containing 10 mg. per ml., was added without waiting for the result of the titration, and the pH of the resultant mixture adjusted to between 5·5 and 6·0 by the addition of a little concentrated HCl. After standing for about twenty minutes, an aliquot was taken and tested for completeness of adsorption by centrifuging a small sample of the suspension and titrating the supernate for haemagglutinin. The bulk of the suspension was then centrifuged and the cloudy supernate discarded. Elution of the haemagglutinin from the $AlPO_4$ was carried out with 1 M NaCl buffered, this time, with 0·1 M Na_3PO_4, and using the same volume of fluid as employed for the extraction of the bacteria. Dispersion of the $AlPO_4$ by means of the stirrer was carried out quickly and with care to avoid excessive frothing. The mixture was centrifuged at once for one hour. The supernatant was separated and titrated for haemagglutinin content, and the deposit of $AlPO_4$ and bacterial debris rejected. The haemagglutinin was then adsorbed on a fresh lot of $AlPO_4$. If the titre of the extract was 1/640, an equal volume of $AlPO_4$ was added; if the titre was greater or less than 1/640, the amount of $AlPO_4$ added was adjusted accordingly. The pH was adjusted to 5·5, and the suspension allowed to stand overnight. Finally the mixture was centrifuged, the supernatant discarded, the $AlPO_4$ carrying the haemagglutinin resuspended in physiological saline to 10 mg. per ml., and merthiolate added to 0·01 p.c. Each step in the process was done aseptically and the final product checked for sterility.

Erythrocytes.

Sheep erythrocytes, from citrated or defibrinated blood or from blood preserved with modified Alsever's solution (Bukantz, Rein and Kent, 1946) were used. The cells were sedimented on the centrifuge and washed in three changes of physiological saline immediately prior to use.

Haemagglutinin titrations.

Haemagglutinin titrations were carried out as described by Keogh and North (1948), but the cysteine hydrochloride was omitted from the saline used for the preparation of the dilutions.

Antihaemagglutinin titrations.

Keogh (1947) found that some human sera contained a non-specific lipoid inhibitor of haemagglutinin which interfered with antihaemagglutinin determinations carried out by the method described by Keogh and North (1948). In order to overcome this difficulty, use was made of the finding (Fisher and Keogh, 1950) that erythrocytes sensitized with certain bacterial products, e.g. pertussis haemagglutinin, are susceptible to haemolysis in the simultaneous presence of antibody specific to the agent adsorbed to the red cells, and complement. The haemolytic method of antihaemagglutinin titration was then adopted for the testing of all sera, as it was found to give easily reproducible results, although the sera of laboratory animals did not appear to contain non-specific inhibitor. The details of the method are as follows:

Sensitization of erythrocytes. Culture supernatants with haemagglutinin titres of not less than 16 were used; in some cases it was found necessary to add a little of a 30 p.c. aqueous solution of sodium chloride to eliminate a slight haemolytic effect of the hypotonic supernate. Erythrocytes (1 part) were suspended in excess (100 parts) supernate; after 10–15 minutes standing on the bench, the cells were spun out, washed twice in saline and resuspended in saline to the desired concentration.

Sera. Sera from guinea-pigs and rabbits were inactivated at 56° C. for 25–30 minutes. Mouse sera, obtained either from tail vein bleedings or from exsanguination of the animals from the axillary vessels under anaesthesia, were not inactivated as they do not contain complete haemolytic complement for sheep erythrocytes; moreover, it was found that the antibody in mouse sera, particularly if present in low titre, became difficult or impossible to demonstrate after inactivation. All sera were absorbed with washed sheep cells equal in volume to the undiluted serum, in order to remove natural haemolysins. The absorptions were carried out on the bench for 15–20 minutes.

Complement. Guinea-pig serum, not more than 24 hours old, was absorbed in the ice-water bath with an equal volume of sheep erythrocytes, in order to remove natural haemolysins.

Technique of the tests.

Doubling dilutions of the sera were prepared in saline, mixed with sensitized cell suspension and incubated at 37° C. for 15–30 minutes. Complement was then added to each tube, the contents mixed and the tests placed in the 37° C. water-bath until the titration of a reference rabbit serum, always included with titrations of unknown sera, had developed haemolysis to the accustomed serum dilution; the time required for incubation was 40–60 minutes. Controls, to all of which complement was added, were: normal cells in the first dilution of each serum; normal cells in saline; and sensitized cells in saline.

The volume of serum per tube, and the concentrations of cells and complement, depended on the amount of serum available. The following two schedules of volumes and concentrations, giving identical results on the reference rabbit serum, were used:

(i) 10 drops serum dilution, 1 drop 5 p.c. cells, 1 drop 1:2 complement (in Wassermann tubes);

(ii) 1 drop serum dilution, 1 drop 1 p.c. cells, 1 drop 1:3 complement (in Dreyer tubes).

Antihaemagglutinin titres are expressed as the reciprocal of the highest serum dilution in which complete lysis was observed. In the tests set up with 1 drop lots of the ingredients, the figures refer to serum dilutions obtained after the addition of the erythrocyte suspension.

Immunization of mice.

The immunizing agents were injected by the intraperitoneal route.

Challenge of immunized mice.

Suspensions of organisms of strain P61 were instilled intranasally by the method of Burnet and Timmins (1937).

RESULTS.

Antigenicity of adsorbed haemagglutinin.

In mice. Groups of mice were immunized with single intraperitoneal injections of graded amounts of the preparation. Some of the animals were challenged with living organisms for the demonstration of protective immunity (see below). The other mice were used to determine antihaemagglutinin levels at intervals over a period of seventeen weeks; for each immunizing

dose, five mice were bled at the desired times, the sera pooled and the titrations carried out on the pooled specimens.

Doses of 1·0 ml. and 0·5 ml. were toxic for mice; 36 p.c. and 24 p.c. of the respective groups died during the first week and most of the survivors were severely affected as judged by their distended abdomens and somewhat sluggish movements.

The results of the antihaemagglutinin titrations are shown in Table 1. High antihaemagglutinin titres were present in the sera of the groups receiving 0·125 ml. or more of the product; on the lowest dose, titres were still measurable. The maximum titres were observed 3 weeks after immunization; some fall had occurred by the time the last specimen was obtained.

TABLE 1.

Antihaemagglutinin titres of pooled mouse sera following injection of a single dose of adsorbed haemagglutinin.

Immunizing dose per mouse	Time in weeks between immunization and bleeding				
	1	2	3	6	17
1·0 ml.	4	256	128	128	32
0·5 ml.	8	64	128	128	64
0·25 ml.	8	64	128	128	64
0·125 ml.	4	64	128	128	■
0·06 ml.	4	16	32	16	8
Nil (controls)	<2	<2	<2	<2	<2

■ = No specimen available.

In guinea-pigs. Table 2 shows the result of antihaemagglutinin titrations carried out on the sera of guinea-pigs injected with various doses and bled at intervals over a period of 6 months. Two guinea-pigs were used for each dose and the titrations carried out on individual serum specimens.

TABLE 2.

Antihaemagglutinin titres in guinea-pig sera following injection of a single dose of adsorbed haemagglutinin.

Immunizing dose per guinea-pig	Time in weeks between immunization and bleeding				
	0	2½	5	9	27
1·0 ml.	<5	80	80	40	■
	<5	■	320	160	40
0·5 ml.	<5	160	320	160	■
	<5	80	160	40	20
0·25 ml.	<5	80	■	■	■
	<5	80	80	40	■
0·125 ml.	<5	20	40	10	5
	<5	20	20	10	5

■ = no specimen available.

The response was similar to that observed in the mice, both as regards antibody level and duration.

Protection of mice against intranasally introduced infection with H. pertussis.

By active immunization. The groups of mice whose antihaemagglutinin titres are shown in Table 1, were drawn upon to provide material for this experiment. Two weeks after the injection of the immunizing dose, 40 mice were taken from each group and challenged, giving half the animals 10 million, and the other half 20 million organisms. The results are shown in Table 3. The best protection was shown by the group receiving 0·25 ml. adsorbed antigen; this was in accordance with the observation that higher doses were markedly toxic (see above). The groups receiving the lowest immunizing dose (0·06 ml.) still showed significant protection compared with the controls.

TABLE 3.

Active immunization of mice with adsorbed haemagglutinin.

Groups	Immunizing dose (ml. per mouse)	Anti-haemagglutinin titre of pooled sera	Challenging dose (millions of organisms per mouse)	Survivors	p.c. Survivors
A.	1·0	256	20	3/13 } 11/26	42
			10	8/13 }	
B.	0·5	64	20	7/16 } 11/32	34
			10	4/16 }	
C.	0·25	64	20	9/20 } 19/40	47
			10	10/20 }	
D.	0·125	64	20	4/20 } 12/40	30
			10	8/20 }	
E.	0·06	16	20	1/20 } 9/40	22
			10	8/20 }	
F.	Nil (controls)	<2	20	0/20 } 1/40	2
			10	1/20 }	

The difference between the survival rates in Groups E. and F. is statistically significant ($P = 0·0066$).

By passive immunization. Intraperitoneal injection of 0·2 ml. serum, containing anti-haemagglutinin to a titre of not less than 320, protected mice significantly against intranasal challenge administered four hours after the immunizing dose. This result was obtained in several experiments in two of which the protective serum used was that of mice immunized with adsorbed haemagglutinin.

Effect of heating and shaking on soluble (extracted) and adsorbed haemagglutinin.

The haemagglutinin of pertussis supernatants has been shown to lose its erythrocyte agglutinating power rapidly when heated over 50° C. (Keogh, 1947), and also on shaking (Fisher, 1949), while its antigenic and antibody fixing capacities were found less susceptible to destruction (Fisher, 1950).

In preliminary tests, it was found that adsorption of the antigen to aluminium phosphate increased its stability considerably towards heat and agitation. Samples of adsorbed antigen kept in the water-bath at 56° C. for two hours and subsequently eluted and tested for haemagglutinin activity, showed no loss of titre; the erythrocyte-agglutinating power of soluble haemagglutinin was completely destroyed by this treatment. After heating the adsorbed

haemagglutinin for three hours all dilutions of the eluate gave only weak agglutination patterns; eluates of adsorbed haemagglutinin heated for shorter periods haemagglutinated red cells strongly. Immersion in a boiling water bath for 5 minutes destroyed the haemagglutinin completely whether adsorbed or in solution.

The effects of heating and of shaking on the antigenic power of extracted (soluble) and adsorbed haemagglutinin were then investigated.

A sample of adsorbed haemagglutinin was divided into two parts. In one of these the antigen was eluted from the aluminium phosphate with alkaline 1 M sodium chloride. Portions of both the adsorbed and eluted haemagglutinin were heated in the water-bath at 56° C. for two hours; another portion was agitated violently with glass beads on a "Microid" flask shaker for 30 minutes, rendering the soluble antigen cloudy; and some was kept for a control. Groups of five mice were then injected intraperitoneally with graded amounts of these materials. Following the injections, some of the animals died, presumably due to the toxic effects of the injected material. The surviving mice were bled three weeks later, sera from those given the same dose pooled, and titrated for antihaemagglutinin content.

TABLE 4.

Effect of heating and of shaking on the antigenicity of soluble (extracted) and of adsorbed haemagglutinin: antihaemagglutinin titres of pooled mouse sera.

State of haemagglutinin		Soluble			Adsorbed		
Treatment		Nil	Heating	Shaking	Nil	Heating	Shaking
Immunizing dose per mouse (ml.)	1	■(5)	■(5)	■(5)	128(2)	64(3)	128(1)
	0·5	128(1)	16	64(2)	128	64(3)	128
	0·25	128	16	64	256	128(1)	64(1)
	0·125	64	16	32	64	64	32
	0·06	32	<2	<2	32	64(1)	64
	0·03	8(1)	<2	<2	8	16	16
	0·015	2	<2	<2	4	8	2
	0·007	<2	<2	<2	<2	<2	<2

■ = no specimen available.

The figures in brackets indicate the number of mice which died during the three weeks period between the immunizing injection and the bleeding.

The results are shown in Table 4. All the mice (15) injected with 1·0 ml. of soluble haemagglutinin died during the course of immunization; the corresponding dose of adsorbed haemagglutinin killed 6 animals; a few deaths also occurred on lower doses of both the soluble and the adsorbed antigens. The difference in the survival rates of the groups receiving 1 ml. antigen was statistically significant ($P = 0{\cdot}0003$). The adsorbed product, therefore, appeared significantly less toxic than the soluble haemagglutinin. The antihaemagglutinin titres indicated that heating and shaking markedly impaired the antigenic power of soluble haemagglutinin, and this was particularly noticeable when the dose was below that producing the maximum effect, e.g. 0·06 ml. per mouse or less. The adsorbed antigen did not appear materially affected by either heating or shaking.

The presence of bacterial agglutinogen in extracted haemagglutinin.

The erythrocyte-adsorbable antigenic complex of pertussis supernatants has been found to contain a small amount of bacterial agglutinogen (Fisher, 1950). Evidence that extracted haemagglutinin also contained similar antigenic material is provided by the following test.

Eight rabbits were immunized with 4 doses of 0·5 ml. adsorbed haemagglutinin given at fortnightly intervals. Pooled bleedings obtained prior to immunization and a week after the last injection gave the following titres:

	Antihaemagglutinin	*Bacterial agglutinin*
Initial bleeding	<5	<5
Final bleeding	160	160

DISCUSSION.

The extraction of haemagglutinin from organisms of *H. pertussis*, its adsorption on aluminium phosphate, elution of the bacterial fraction, and its readsorption on aluminium phosphate floccules yielded a potent and stable antigen, capable of immunizing mice against intranasally introduced infection with the parent organisms. Protection against the experimental disease was afforded by the prophylactic administration of serum from animals immunized with the same antigen.

Adsorption on aluminium phosphate floccules increased the resistance of pertussis haemagglutinin to destruction by heating or shaking, and reduced its toxicity without loss of antigenic power; the lessened toxicity of the adsorbed antigen was presumably due to its lower rate of absorption. Following administration of the adsorbed antigen, antibody levels in mice were well maintained for periods up to four months; in guinea-pigs, antibody was demonstrable for at least six months. Corresponding tests for duration of antibody have not been carried out with soluble haemagglutinin.

Roux (1888) found that diphtheria toxin could be precipitated from solution with potassium alum. Different compounds of aluminium have since been applied to the precipitation of diphtheria toxoid, the latest being aluminium phosphate (Holt, 1950). Harrison, Franklin and Bell (1938) precipitated pertussis vaccine with alum. More recently, Ungar and Muggleton (1949) reported that virulence and phase 1 characteristics of *H. pertussis* were correlated to their agglutinability by aluminium phosphate and that the same substance was capable of removing a toxic component from pertussis supernatants.

Haemagglutinin extracted from the bacteria appeared to be associated with bacterial agglutinogen; the haemagglutinin in culture supernatants has been reported (Fisher, 1950) to have similar antigenic activity. The relationship of this agglutinogenic component to haemagglutinin has not been elucidated and will form the subject of further study; whether it is identical with phase 1 agglutinogen is at present uncertain. Agglutination of phase 1 organisms by immune sera is a reaction involving, according to Anderson (North, Keogh, Anderson and Williams, 1939), more than one antigen.

SUMMARY.

Haemagglutinin was extracted from *H. pertussis* with alkaline 1 M NaCl and adsorbed on aluminium phosphate at slightly acid pH. The antigen was purified by elution and readsorption.

Haemagglutinin in the adsorbed form was more resistant to destruction by heat or agitation than in solution. Mice immunized actively with the adsorbed antigen, or passively with sera of animals immunized with it, were protected against the lethal effects of intranasally introduced infection with *H. pertussis*.

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STUDIES ON THE OESTROGENIC SUBSTANCE IN SUBTERRANEAN CLOVER: (*TRIFOLIUM SUBTERRANEUM L.* VAR. DWALGANUP)

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Earlier investigations (Bennetts, Underwood and Shier, 1946; and Bennetts, 1946, 1947) on a breeding problem of sheep had shown that the disease was probably due to the presence of some oestrogenic substance in the subterranean clover (*T. subterraneum L.* var. Dwalganup) which was the main species in the pastures grazed. Subsequent work by Curnow, Robinson and Underwood (1948) and Robinson (1949) had demonstrated the presence of considerable oestrogenic activity in the extracts of this clover. The present paper gives the results of experiments designed to obtain further information on the chemical nature of the oestrogen, the preparation of large scale concentrates and the method of assay.

METHODS.

Preparation of concentrates and extracts.

Robinson (1949) used alcohol for the extraction of clover but this method was not found satisfactory for large scale work.

Recent observations by English workers (Legg, Curnow and Simpson, 1950; and Curnow, 1950) had shown that the oestrogenic activity was associated with the "chloroplast" fraction of the press juice of clover. The following procedure, based on these observations, was used on twenty-five hundredweight of clover harvested at flowering in 1949. The freshly cut clover was crushed in a hammer mill and the juice separated by mechanical pressure. The residue was discarded. The juice showed no apparent loss of activity after several weeks when stored at 4° C. and preserved with 0·25 p.c. chloroform. The chloroplast fraction was first separated by centrifuging at 30,000 r.p.m. in a Sharples super-centrifuge. The solid material was dried in a stream of air at 55° C. The juice, from which the bulk of the chloroplasts had been removed, was then heated to 70° C., the precipitate filtered and dried in a similar manner. The two products had similar levels of activity and kept well in the cold.

As some larger scale extractions (100 gm. lots) had given poor recoveries, the finely ground, dried products were extracted in small lots (less than 20 gm.) with normal alcoholic sodium hydroxide. Three extractions were given with 150–200 ml. portions of NaOH and the mixture refluxed for 10 minutes each time. After neutralization and evaporation, the

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oestrogen was separated by ether extraction from a sodium carbonate-bicarbonate solution as described under the method for assay. The oestrogenic material was separated from neutral substances by extraction from ethereal solution with 0·1 N sodium hydroxide. This product, which represents about 3 p.c. of the original chloroplasts, is referred to as the "crude phenols."

The "crude phenols" contain a fairly large proportion of fatty acids and towards the end of the investigation it was found that these acids could be separated from the oestrogen by esterification with methyl alcohol and sulphuric acid.

The "crude phenols" were refluxed for 30 minutes with anhydrous methyl alcohol containing 1 p.c. (w/v) sulphuric acid at the rate of 100 ml. of methyl alcohol per gram of extract. After cooling, the mixture was neutralized with alcoholic sodium hydroxide (pH 6·7, brom-thymol blue) and evaporated to dryness under vacuum. Water was added to dissolve the sodium sulphate, the solution acidified, and the mixture extracted with ether. The ether extract was evaporated to a convenient volume and the phenols extracted three times with equal volumes of 0·1 N NaOH. The neutral substances contained no oestrogenic activity and were discarded. The alkaline extract was acidified and extracted with ether which was washed with freshly prepared, saturated $NaHCO_3$, then with water until neutral and evaporated to dryness. This extract, which represents 25–40 p.c. of the "crude phenols", was a clear brown viscous oil and is referred to as the "purified phenols" fraction.

Method for assay of clover and clover extracts.

Attempts have been made to improve further Robinson's (1949) method for the preparation of extracts for assay of clover samples. The following is the best method obtained. Green clover (50–100 gm. fresh weight) is collected directly into absolute alcohol (400 ml.) and stored at 4° C. until extracted. The clover is boiled with fresh alcohol for one hour. The two extracts are bulked, concentrated under vacuum to about 250 ml. and boiled for 10 minutes with one fifth volume of 3 N alcoholic NaOH. Following alcoholic extraction, the clover leaves are disintegrated in a high speed macerator, boiled for 10 minutes with 0·5 N alcoholic NaOH, filtered and boiled again with alcohol. The combined extracts are neutralized with 20 p.c. alcoholic H_2SO_4 to pH 6·7 (brom-thymol blue) and allowed to stand overnight at 4° C. After filtration, the solution is evaporated to dryness under vacuum. Purified "white spirit" (Beck, 1950) is added as anti-frothing agent if necessary. The dried residue is allowed to stand with 50 ml. freshly prepared, saturated $NaHCO_3$ and 50 ml. ether, any remaining solid residue dissolved in a minimum amount (10–20 ml.) of 4·5 p.c. Na_2CO_3 and a further 30 ml. of $NaHCO_3$ added. The oestrogen is extracted with 6 additional lots of 100 ml. ether. If any solid material separates out during the extraction it is redissolved as above. The ether extract is concentrated and the active fraction removed by three extractions with equal volumes of 0·1 N NaOH. The alkaline solution is acidified and the oestrogen re-extracted with ether. After washing with water the ether phase is evaporated to dryness.

In order to determine the loss of oestrogenic activity during the extraction and purification processes, two lots of a non-oestrogenic species (*Medicago sativa*) were extracted in the usual way. To one a measured amount of standardized chloroplast extract was added before extraction and to the other the same amount was added after the extraction and purification were complete. The activities of the two extracts were then compared and it was found that the loss of activity was 27 p.c. (Limits 14 and 39 p.c., $P = 0{\cdot}05$.).

The method of assay of the clover or chloroplast extracts was a modification of that described by Evans, Varney and Koch (1941). Entire immature female mice (7·5–10·5 gm.) were randomized into groups so that littermates were distributed evenly. Groups of six were generally used but greater accuracy was obtained when this was increased to ten. The log. dose-response line of the clover extracts was found to be significantly different from those of

oestrone and oestradiol so that it was necessary to prepare a large amount of "crude phenols" extract for use as a reference standard. This was done by extracting small batches (10–20 gm. dry weight) of the chloroplast material by the method detailed above. This standard is best kept in the dry state under nitrogen, as alcoholic solutions show a slow loss of potency.

Generally extracts to be compared were assayed at two or three dose levels of both standard and unknown. It was necessary to have doses differing by a factor of 2 or 3 as the slope of the log.-dose response line is rather low. The mice were injected subcutaneously twice daily for three days with 0·05 ml. of a peanut oil solution of the extract under test. They were killed 18 hours after the last injection and the uteri dissected out and fixed in Bouin's fluid for 24 hours. The uteri were pressed between filter papers and weighed.

The mean uterine weight of the untreated controls was 2·5–3·5 mg. and the maximum obtainable by clover extracts a little over 20 mg., but the dose-response line is only linear in range 6–15 mg., and hence it is necessary to adjust the doses so that the responses will fall within this range. Another feature of the responses obtained with clover extracts is that, if the doses are increased above a certain level, the responses progressively decline though not to the control level. This phenomenon has also been noted by Costello and Lynn (1950) in assays of oestrogenic licorice root extracts by the Allen and Doisy (1923) vaginal smear technique. Thus it seems that this phenomenon is related to the crude nature of plant extracts rather than to the assay method.

The results obtained by the above assay method were analysed by the statistical methods described by Emmens (1948). The fiducial limits of error found were often rather wide, and this must be kept in mind in interpreting the results. These limits, however, could be narrowed by the use of larger numbers of animals per test.

RESULTS.

Although the extracts are relatively crude, it is possible to obtain a considerable amount of information on the nature of the oestrogen by phase distribution studies and by following the chemical distribution of biological activity after treatment with a number of comparatively mild chemical reagents.

TABLE 1.

Partition coefficients of clover oestrogen.

Phases	Percentage in aqueous phase		
	Clover oestrogen	Oestrone; oestradiol	Oestriol
Ether:water	4	(0)	4
Ether:$NaHCO_3$ (Sat.)	0	0	0
Ether:Na_2CO_3 (9 p.c.)	78 (64–88)	2	20
	88* (85–90)	—	—
Ether:0·1 N NaOH	96	33	97
Ether:N NaOH	98	62	100
Benzene:Water	6·5 (5–8)	0	78
	8·7* (7–11)	—	—

* These separations done on "purified phenols", all others on "crude phenols".

Partition coefficients of the clover oestrogens.

The results obtained from phase separations done on "crude phenols" and in two cases on the "purified phenols" are set out in Table 1. Where the results were suitable for analysis, the statistical limits of error ($P = 0{\cdot}05$) have been calculated from the figures and are given in brackets below the respective means. Corresponding partition coefficients for oestrone, oestradiol and oestriol (Bachman and Pettit, 1941) are given for comparison.

Chemical studies on the clover oestrogen.

The activity is not precipitated with digitonin and accordingly the oestrogen is not a 3β hydroxy steroid. Condensation with Girard's reagent T leaves the activity in the non-ketonic fraction. The fact that concentrated hydrochloric acid does not dissolve the activity from the "crude phenols" suggests that the γ pyrone ring is absent.

Treatment of the "crude phenols" with acetic anhydride and pyridine results in the conversion of at least 80 p.c. of the activity into a form no longer extracted from ether by 0·1 N sodium hydroxide. This indicates that the acidic groups of the oestrogen are phenolic. Continued shaking of the ethereal solution with 0·1 N NaOH extracts a considerable amount of activity, indicating a fairly rapid hydrolysis. The acetylated product has an activity as great as, or slightly greater than the original but further investigations were not carried out because of its ease of hydrolysis and because of the large amount of ether-insoluble and oil-insoluble material present.

Benzoylation by the standard Schotten-Baumann reaction gives an alkali-insoluble product showing a low oestrogenic activity but the product is largely insoluble in ether and oil.

The "crude phenols" react slowly with diazomethane to give a product of lower oestrogenic activity in which the active fraction is much less alkali-soluble than in the original material. Methylation of this material in acetone solution with methyl sulphate and sodium hydroxide gives a three to four fold rise in oestrogenic activity (one batch gave a ratio of 3·6 with limits of 2·4 and 5·3, $P = 0{\cdot}05$) and the activity is now in the alkali-insoluble fraction. A product of identical properties and biological activity is obtained by direct methylation of the "crude phenols" with methyl sulphate.

The "purified phenols" react similarly with diazomethane but further methylation with methyl sulphate gives a smaller rise in activity. Direct methylation of the "purified phenols" with methyl sulphate gives a product of low activity. It is suggested that the acids present in the "crude phenols" exerted a protective action against the strong alkali used in methylation and that some destruction of the oestrogen has occurred with the purer material.

The changes in alkali solubility after methylation may be explained on the hypothesis that the oestrogen is a dihydric phenol. Diazomethane would appear to methylate completely one phenolic group with incomplete methylation of the other group while methyl sulphate reacts with both groups to give the dimethyl ether.

An accurate comparison of the oestrogenic potency of the original and methylated products is not possible as the slopes of the log. dose-response lines are different. Fig. 1 shows the dose-response curves of a batch of "purified phenols" and the product obtained by methylation of the same material first with diazomethane and then with methyl sulphate. Curves for oestradiol and for another batch of "crude phenols" are given for comparison. These last two lines were obtained at different times from the first two and so may not be strictly comparable. The curves of the "purified phenols" and to a lesser extent of the methylated product have flattened out at somewhat lower levels than usually occur with "crude phenols". It will be noted that with the particular assay method, the methoxy compound appears to have a lower relative activity than the parent phenol at low levels of injection but this is reversed at higher levels.

Data quoted by Solmssen (1945) suggest that the conversion of the partly methylated product to the fully methylated compound should be accompanied by a decrease of oestrogenic activity. The observed rise may be due to the action of the methyl sulphate or sodium hydroxide, or both on some group other than the phenolic hydroxyl groups. On the other hand the increased activity of the fully methylated product may be due to a decreased absorption rate or to a greater resistance to inactivation in the body of the assay animal.

As diazomethane normally methylates only carboxylic acids and phenols, an attempt was made to ascertain whether any free alcoholic groupings were present in the methoxy compound formed by diazomethane. The standard condensation with succinic anhydride and pyridine (Pincus and Pearlman, 1941) was carried out but no activity was found in the succinic half-ester (alcohol) fraction.

A study of the methoxy derivatives suggests that a lactone group is not present in the original oestrogen. The methoxy compound formed by diazomethane is refluxed for 10 minutes with 2·5 N alcoholic NaOH and then poured into boiling 2·5 N aqueous NaOH. This treatment would normally open a lactone ring and give a sodium salt insoluble in benzene. However, the bulk of the oestrogenic potency can be extracted from the alkaline solution with benzene. If the benzene-extracted solution is acidified and extracted with ether, the small amount of oestrogen obtained behaves like a phenol i.e. it is extracted from ether by 0·1 N NaOH. If it were a lactone the acidification would be expected to close the ring and the product would not be extracted by NaOH. Furthermore, in the methylation by methyl sulphate, the strong alkali used (40 p.c.) would normally open a lactone ring and the methylated product would give a free carboxylic acid on hydrolysis. Tests have shown that after hydrolysis the oestrogenic fraction is completely insoluble in alkali. This fact also gives conclusive evidence that a carboxylic acid group is absent.

The methyl ether formed by methyl sulphate is not volatile in steam.

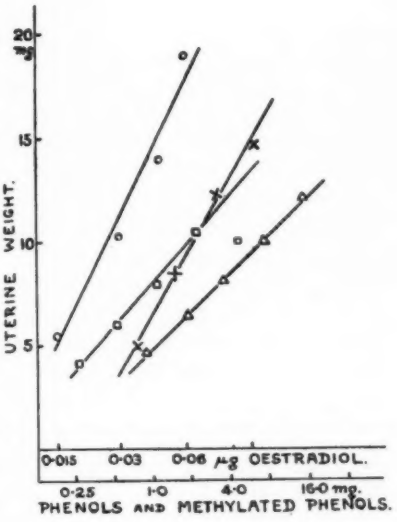

Fig. 1. Dose-response lines for oestradiol, and for oestrogenic preparations from subterranean clover.

—o———o— Oestradiol:
$E = 21{\cdot}17X + 106{\cdot}9.$

—△———△— "Crude phenols":
$E = 6{\cdot}182X + 4{\cdot}74.$

—□ —— □— "Purified phenols":
$E = 7{\cdot}315X + 7{\cdot}91$ (omitting 4·45 mg. dose).

—×———×— "Purified phenols" after complete methylation:
$E = 10{\cdot}98X + 6{\cdot}41.$

Where E = observed uterine weight in mg.
$X = \log_{10}$ of total dose in mg. per mouse.

DISCUSSION.

Work by Robinson (1949) on whole clover showed that alcoholic sodium hydroxide produced extracts of higher oestrogenic potency than alcohol alone. Furthermore, saponified extracts are more soluble in the oil used for injection into the test animals, and because of these facts, we have used saponified material throughout in this investigation. It is pertinent to consider here whether such treatment may have altered the oestrogen molecule.

We have confirmed Robinson's observation on the lower activity of alcoholic extracts as compared with those of alcoholic sodium hydroxide; in one test the ratio of activity was 0·46 (limits of 0·28 to 0·76, P = 0·05) but this difference is not great when the limitations of the assay method are taken into consideration. It has been shown by Emmens (1939) that palmitic and other fatty acids can greatly increase the oestrogenic activity of oestriol and it is probable that the high proportion of fatty acids in saponified extracts may explain at least part of the increased activity of such extracts. It is also possible that in the clover plant the oestrogen is linked to other compounds which are split off during hydrolysis, and this also could account for the change in activity.

It would seem that the "crude phenols" are relatively stable toward boiling 0·5 N alcoholic sodium hydroxide, as the oestrogenic potency does not alter significantly if the time of hydrolysis is increased from 10 minutes to 30 minutes.

The relatively small change in activity which occurs during hydrolysis suggests that such treatment does not produce any radical change in the oestrogen molecule.

Although this investigation has not given sufficient evidence to suggest the identity of the clover oestrogen, it would appear that the compound is not one of the common steroid oestrogens. The partition coefficients show definitely that it is not oestrone or oestradiol. Although there is a slight resemblance to oestriol, the clover oestrogen appears to be more acidic and less hydrophylic than this compound. The evidence from the methylation studies suggests that at least two phenolic groups are present. The partition coefficient between benzene and water shows that the number of hydroxyl groups per molecule must be limited and if a comparison with oestriol is valid, the number will be less than three. It is accordingly suggested that the molecule contains two phenolic groups. A fairly large range of oestrogenic, dihydric phenols is recorded in the literature, but unfortunately, no partition coefficients seem to be available for comparison with the clover oestrogen.

Before further progress in identification can be made, it will be necessary to effect very considerable purification of the so-called "purified phenols".

SUMMARY.

Details are given for the large scale preparation of oestrogenic extracts from subterranean clover (*T. subterraneum L.* var. Dwalganup). The process involves the preparation of press juice, the separation of chloroplasts and of the heat-precipitable fraction of the juice. The dried chloroplast and heat-precipitated materials are extracted with alcoholic NaOH, and the oestrogenic material extracted with ether from an aqueous Na_2CO_3–$NaHCO_3$ solution of the extract.

Phase distribution studies, methylation and acetylation tests on the purified extract have indicated that the oestrogen probably contains two phenolic groups. Tests have failed to show the presence of ketone, lactone, carboxylic, or alcoholic groupings.

Some details of the assay method are given.

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DIGESTION OF PROTEIN IN THE ALIMENTARY CANAL

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It is generally recognized that pepsin, trypsin and chymotrypsin split proteins to form peptides. Succus entericus obtained from the duodenum and small intestine of cats following various neural sections and stimulation was found to be free of peptidases when made cell-free by centrifugation (Wright *et al.*, 1940). The epithelium of the small intestine is known to be rich in peptidases. These observations suggest that proteins are broken down by gastric juice and pancreatic juice to peptides and that these peptides are further digested within the epithelial cells of the small intestine. Such a mechanism is so different from the classical view that it could not be accepted without more direct evidence than that given.

A dog was prepared with a ⊔-shaped enterostomy with the proximal end of the lower segment brought out through the wall of the abdomen. The end-to-side anastomosis was twelve inches from the pancreatic duct. The animal was trained to lie on the table and a fine catheter was passed down into the stream of intestinal contents one and a half hours after a meal of raw meat, the animal having been fasted for the previous twenty-four hours.

The material obtained by aspiration was prepared for partition chromatography on filter paper by adding 4 vols. absolute alcohol to 1 vol. intestinal juice. The precipitate was sedimented by centrifugation, the supernatant concentrated *in vacuo* to original volume and desalted by the electro-dialysis method of Consden, Gordon and Martin (1947). The material was then concentrated × 7·5 *in vacuo* and run in phenol and then *S. collidine*, and the colour developed with ninhydrin (·1 p.c. solution in butanol). The chromatogram (Fig. 1) showed eighteen reactive areas.

The protein-free material was subjected to acid hydrolysis and treated as for the unhydrolysed material. Hydrolysis was carried out by 6N HCl in a sealed vessel at 105° C. for 24 hours. The chromatogram showed increased concentration of all previously recognized amino acids and the disappearance of none of the ninhydrin reacting spots. The unrecognized substances 17 and 18, though in a position characteristic of peptides, remained stable. This fairly full spectrum of amino acids and the absence of any spots which from their position, colour reactions or disappearance on hydrolysis, could suggest that they were due to peptides, was surprising at such a short distance from the duodenum. The impression of increased concentration of amino acids after hydrolysis was checked by formol titration and gave an increase from 95·2 mg. (NH_2) nitrogen per 100 ml. to 170·8 mg. (NH_2) nitrogen per 100 ml. Apparently, therefore, there are peptides (or other combined forms of amino acids) in the juice but they are not apparent on the chromatograms.

[1] This work was carried out with the assistance of a grant from the National Health and Medical Research Council.

The possibility that the amino acids detected were present in the meat which had been eaten was tested by homogenising a portion of the beef in a Waring Blendor with three times its weight of distilled water and treating as for the jejunal juice. Only eight faint spots appeared and these were:

1. Glutamic acid
2. Serine
3. Glycine
4. Taurine
5. Unknown
6. Alanine
7. Unknown: Gives a brownish colour—moves a little more rapidly than histidine. It could be a histidine peptide such as carnosine or anserine. It could also be methyl. histidine.
8. Valine

It was clear that the appearances in the jejunal juices were not due to autolysis of the beef.

The next possibility was that the amino acids had appeared as a result of acid hydrolysis in the stomach. Homogenised muscle was therefore digested for four hours at 37° C. with HCl added to pH 2. Alcohol failed to precipitate all the protein so the supernatant from the alcohol treatment was concentrated *in vacuo* to the original volume and twice the volume of acetone added. Further protein was precipitated and the supernatant after centrifugation was treated in the same way as other specimens. The chromatogram was the same as for the undigested beef except for:

1. Trace of aspartic acid.
2. Disappearance of spot No. 5 previously seen under taurine.
3. Appearance of leucine.
4. Appearance of a spot, probably arginine.

It was therefore proven that jejunal juice of the dog after a meal of beef muscle contains amino acids equivalent to half the total amino-nitrogen present and that these had not resulted from autolysis in the beef nor from acid hydrolysis. It became important, therefore, to investigate whether they might have resulted from the action of gastric or pancreatic juice.

Gastric juice was obtained from the washed stomach of a decerebrate cat by vagal stimulation supplemented by subcutaneous histamine. Pancreatic juice was obtained by the use of intravenous secretin in a nembutalised cat to which eserine 0·06 mg. and acetylcholine 2 mg., each subcutaneously, had been given. The substrate was egg white spun into boiling distilled water to provide threads of denatured protein.

A sample of each juice and two of the egg white, one in N/10 HCl and one in N/10 $NaHCO_3$, were incubated at 37° C. for six hours and prepared for chromatography. The chromatograms showed no discrete areas recognizable as amino acids or peptides.

To 20 ml. of gastric juice 3 gm. of denatured egg white were added and the mixture incubated at 37° C. Aliquots were taken at 1, 3, 8 and 11 hours and treated in the same way as the jejunal juice. The chromatograms showed the progress of digestion from large peptides[2] moving rapidly in phenol to a mixture of amino acids and peptides in the last two specimens. But peptides were in marked excess of amino acids even in the 11-hour specimen.

A portion of the material removed at eight hours was freed of particles and neutralized by adding N/10 NaOH. An equal volume of activated pancreatic juice was added giving a pH 8. The mixture was incubated for eight hours and prepared for chromatography. The chromatogram (Fig. 2) showed a complete range of amino acids with no evidence of peptides. Acid hydrolysis of this material, however, caused an increase in all the amino acids present estimated chromatographically at about 2–3 times the original concentration.

These experiments indicated that the presence of the amino acids in the jejunal juice could have resulted from the action of pancreatic juice on the gastric digest of the beef muscle. It appeared possible, however, that the pancreatic digestion of gastric juice might have contributed to the result. Gastric juice was, therefore, incubated at 37° C. for six hours. It was

[2] For description of the movement of protein and macro molecular peptides in chromatography, see Wynn and Rogers (1950).

then neutralised with N/10 NaOH and to two volumes three volumes of pancreatic juice were added. After incubation at 37° C. for eight hours the digest was prepared for chromatography and gave a complete spectrum of amino acids without evidence of peptides. Pancreatic digestion of gastric juice could therefore have contributed to the result.

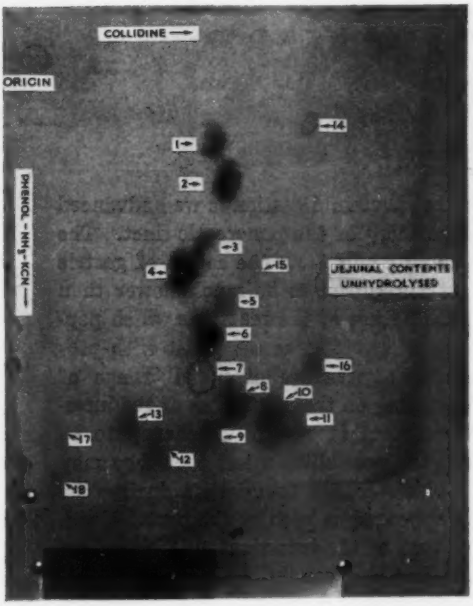

Fig. 1.

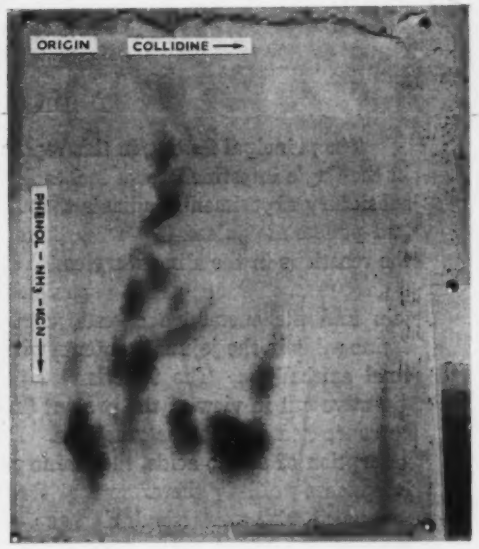

Fig. 2.

Fig. 1. Chromatogram of unhydrolysed jejunal protein-free fluid.

1. Aspartic acid	7. Oxyproline	13. Lysine
2. Glutamic acid	8. Valine	14. Cysteic acid
3. Serine	9. Proline	15. Taurine
4. Glycine	10. Leucine (s)	16. Tyrosine
5. Threonine	11. Phenylalanine	17. Unknown
6. Alanine	12. Arginine	18. Unknown

Fig. 2. Chromatogram of supernatant from denatured egg albumin subjected first to digestion by gastric juice at pH 2 and then to pancreatic juice at pH 8.

A small amount of pure trypsin and pepsin (Armour) was available. The following experiments were done to see whether the amino acids obtained from pancreatic digestion of gastric juice resulted from digestion of pepsin by trypsin or from digestion of other proteins in the gastric juice, or whether it was due to presence of peptidases in the pancreatic juice. In 0·2 ml. HCl—sodium acetate buffer, pH 2, 25 mg. of pepsin were dissolved and incubated. Chromatography showed that the protein (enzyme) moved freely in phenol and no amino acids were detected. To 2 ml. of Na_2HPO_4 buffer, pH 8, 25 mg. of trypsin were added and incubated four hours. Chromatography with phenol showed no amino acids in this solution. The pepsin solution was then added to the trypsin solution and pH adjusted to 8.

After incubation at 37° C. for eight hours chromatography showed a few amino acids, but peptides predominated. This suggests that the amino acids present in the pancreatic juice digest of either denatured egg albumin or gastric juice are due to the peptidases in the pancreatic juice.

The reverse experiment where the two enzymes were incubated at pH 2 also showed mainly peptides as the result, but the following amino acids were demonstrated:

1. Aspartic acid
2. Glutamic acid
3. Glycine
4. Alanine
5. Valine
6. Leucine
7. Phenylalanine
8. Tyrosine
9. Lysine
10. Arginine

DISCUSSION.

The principal finding in this report is that protein digestion is well advanced in the dog's intestine twelve inches from the entry of the pancreatic duct. The subsidiary experiments indicate that this could result from the action of gastric and pancreatic juices. *In vitro* these reactions appear to be much slower than the reactions in the alimentary canal, but the nature of the reactions which pepsin, trypsin, chymotrypsin and pancreatic carboxypeptidase carry out with protein and the successive digests, explains the appearance of a full range of amino acids in the jejunal contents without calling on the peptidases of the intestinal epithelium. The function of these enzymes is generally believed to be digestive but in view of the present findings the possibility arises that they may be concerned with other reactions. Dent *et al.* (1949) found that during the absorption of amino acids, the amino acids appeared as such in the portal blood. It appears unlikely therefore that the proteases of the intestinal epithelium are concerned with synthesis during absorption, but this possibility is raised by the observation of Christensen (1947) that feeding glutamic acid to dogs or glycine to humans raises the concentration of conjugated amino acids in the plasma.

SUMMARY.

Jejunal contents taken 12 inches below the entrance of the pancreatic duct of a dog to which meat had been fed, showed a wide range of amino acids.

This degree of digestion could result from the action of gastric and pancreatic juices.

The function of intestinal peptidases remains in doubt.

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THE LIBERATION OF PANTOTHENIC ACID FROM AUTOLYSING YEAST CELLS

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(From the Research Division, Kraft Walker Cheese Co. Pty. Ltd., Melbourne).

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Farrer (1946) has described the liberation from the autolysing yeast cell of aneurin (and cocarboxylase) riboflavin and nicotinic acid. He showed that these vitamins (or co-enzymes) rapidly pass out of the cell once autolysis has commenced and, with brewers' yeast, only three hours autolysis at the optimum temperature (52° C.) is sufficient to permit the passage of the whole of each of these three vitamins into the surrounding medium.

Subsequently (1946–7) some experiments were undertaken with pantothenic acid in the expectation that a similar behaviour would be observed. Such, however, was not the case.

EXPERIMENTAL.

Preparation of yeast samples: Brewers' yeast was received from the brewery as a yeast cream. It was sieved, washed and filtered as described by Farrer (1946).

Autolysis: The procedure was exactly the same as that described earlier (Farrer, 1946).

TABLE 1.

Pantothenic acid media.

Constituent	Pantothenic acid basal medium. (Barton-Wright, 1944)	Modified basal niacin medium (Barton-Wright, 1945)	Constituent	Pantothenic acid basal medium. (Barton-Wright, 1944)	Modified basal niacin medium (Barton-Wright, 1945)
Glucose	2·0 gm.	2·0 gm.	NaOH-treated peptone	0·5 gm.	—
Sodium acetate	1·4 gm.	2·0 gm.	dl Tryptophane	—	0·01 gm.
Acid-hydrolysed casein	0·2 gm.	1·0 gm.	Cystine hydrochloride	0·1 gm.	—
Adenine	1 mg.	1 mg.	l-Cystine	—	0·02 gm.
Guanine	1 mg.	1 mg.	NaCl	0·5 gm.	0·5 gm.
Uracil	1 mg.	1 mg.	$(NH_4)_2\ SO_4$	0·3 gm.	—
Xanthine	1 mg.	1 mg.	Xylose	—	0·1 gm.
Niacin	20 μg.	20 μg.	Inorganic salt solution "A"	0·5 ml.	0·5 ml.
Biotin	0·04 μg.	0·04 μg.	Inorganic salt solution "B"	0·5 ml.	0·5 ml.
Riboflavin	20 μg.	20 μg.	Water (glass distilled)	to 50 ml.	to 50 ml.
Pyridoxine	10 μg.	10 μg.			
Aneurin	—	10 μg.			
p-Amino benzoic acid	10 μg.	10 μg.			

The inorganic salt solutions are as follows:

A. 25 gm. of K_2HPO_4 and 25 gm. of KH_2PO_4 are dissolved in water and made up to 250 ml.

B. This solution contains 10 gm. of $MgSO_4\ 7H_2O$, 0·5 gm. of $MnSO_4.4H_2O$ and 0·03 gm. of $FeCl_3$ dissolved in water, acidified with 5 drops of conc. HCl and made up to 250 ml.

Assay method.

The microbiological assay procedure was used. The organism was *Lactobacillus arabinosus* 17/5 (A.T.C.C. No. 8014) and the medium was that used by Barton-Wright (1944) for niacin, substituting niacin for calcium pantothenate. Actually the same author had given a medium for pantothenic acid (Barton-Wright, 1945) and the two are contrasted in Table 1. There is little difference between them and, as successful assays for niacin were being carried out concurrently, it was considered advantageous to use the one medium for the two assays. The satisfactory results obtained with the pantothenic acid assays demonstrate the success of the niacin medium in determining the former factor.

The standard microbiological assay procedure was used and the result computed by means of the log-log method of Wood (1946–7). As free pantothenic acid was being determined there was no hydrolysis of the two solutions with Mylase P. Pantothenic acid in the form of co-enzyme A or pantothenic acid conjugate is inactive microbiologically.

RESULTS AND DISCUSSION.

Fig. 1 shows the results obtained in a study similar to those made of the other vitamins. The curves are much the same as the earlier ones (Farrer, 1946). There is evidence of a slight lag period preceding autolysis, then there is a rapid liberation of pantothenic acid. Moreover, the influence of the preliminary cold storage of the yeast on the subsequent liberation of the vitamin is again evident. However, there is one important difference, viz. no maximum value is reached and the curve for the fresh yeast shows clearly that the complete liberation of pantothenic acid is a slow process taking much longer than the liberation of the other vitamins. This is confirmed by Fig. 2, obtained with a different sample of yeast, which shows the liberation to be still approaching completion after 24 hours autolysis.

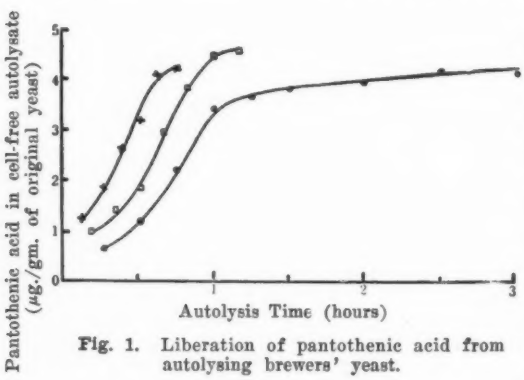

Fig. 1. Liberation of pantothenic acid from autolysing brewers' yeast.

The shape of the pantothenic acid curves resembles that of the free aneurin curves (Farrer, 1946) and suggests an enzymatic liberation of free pantothenic acid from some conjugate. Novelli *et al.* (1949) have discussed this liberation showing that most of the pantothenic acid of living cells occurs as co-enzyme A from which it is split only with difficulty. It seems, however, that there is some free pantothenic acid in the yeast and that this diffuses quickly out of the cell when autolysis begins. Fig. 3 is a first order reaction curve (log $(a—x)/t$) for the results shown in Fig. 2. It was assumed that 12·8 μg./gm. of yeast (the

value obtained at 24 hours) was the maximum value for the yeast. (= "a" in the first order reaction equation $k = l/t \ ln \left(\frac{a}{a-x}\right)$.) This is not so, as later experiments showed, but for the purpose of Fig. 3 it suffices, the main point is that there is a sharp change of direction in the curve at 2 hours which suggests a change in the reaction. This point also coincides with the beginning of the flatter part of the liberation curve and it is suggested that up to this point free pantothenic acid is diffusing out of the cell as part of the breakdown in the normal cell process which begins autolysis. When this is complete the further liberation of free pantothenic acid is dependent on a much slower reaction, viz. the enzymatic liberation of pantothenic acid from the bound form in which it is unavailable to the assay organism.

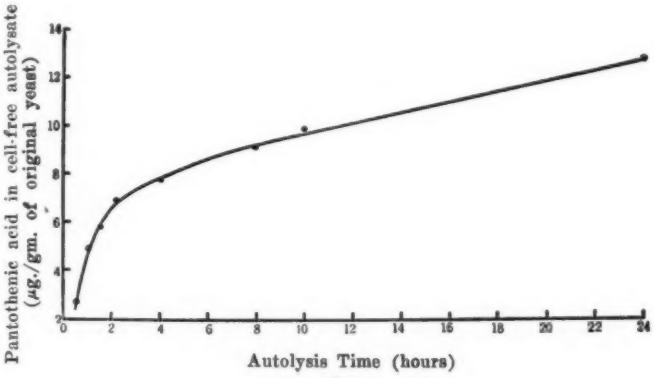

Fig. 2.

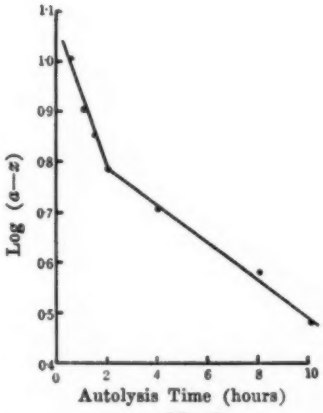

Fig. 3.

Fig. 2. Liberation of pantothenic acid in a 24 hour autolysis of brewers' yeast.
Fig. 3. Log $(a—x)/t$ curve for liberation of pantothenic acid from autolysing brewers' yeast.

Determinations carried out at a later date have used the preliminary digestion with Mylase P, as described by Buskirk *et al.* (1948) to determine total pantothenic acid in yeast and centrifuged autolysate. These have shown that autolysis of brewers' yeast for as long as 45 hours has liberated from the cell as little as 75 p.c. of the total pantothenic acid in the yeast. On the other hand, with other samples of yeast, upwards of 90 p.c. of pantothenic acid has been found outside the cell after 16 hours autolysis. The omission of Mylase P digestion of centrifuged autolysates has given much lower results than those obtained when this hydrolysis has been carried out, and this points to the existence outside the cell of pantothenic acid in the bound form, possibly fragments of coenzyme A as suggested by Novelli *et al.* (1949). Cocarboxylase,

riboflavin and niacin all occur in enzyme systems bound through a phosphate linkage and their rapid and complete liberation from autolysing yeast may be a function of the active phosphatases known to exist in yeast. Indeed the hydrolysis of cocarboxylase to free aneurin has already been attributed to this (Farrer, 1946). However, Lipmann *et al.* (1947) and Novelli *et al.* (1949) have shown that pantothenic acid is involved in two linkages in coenzyme A, one split by a phosphatase, the other by a liver enzyme. It is probably the latter which is responsible for the very slow and apparently incomplete liberation of free pantothenic acid from autolysing yeast.

SUMMARY.

The liberation of pantothenic acid from brewers' yeast autolysing at 50–52° C. has been studied by means of the microbiological assay with *L. arabinosus* 17/5.

In the early stages, up to about 1 hour with fresh brewers' yeast, the course of the liberation resembles the curves obtained with aneurin, riboflavin and niacin, viz. a short lag period preceding autolysis followed by rapid liberation which is virtually a linear function of time. Preliminary storage of the yeast greatly accelerates the liberation to this stage in the subsequent autolysis.

From about 1 hour onwards, however, liberation of pantothenic acid is very slow and does not reach completion (as compared with Mylase P digested yeast) even in 45 hours.

It is suggested that the initial rapid liberation corresponds to the liberation of free pantothenic acid from the cell and that the subsequent slower rate is due to the slow liberation of pantothenic acid from fragments of coenzyme A.

There is evidence for believing that this latter process goes on outside the cell after both bound pantothenic acid and its liberating enzymes have diffused through the cell wall.

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A STUDY OF THE FACTORS INFLUENCING INNERVATION OF MUSCLES BY IMPLANTED NERVES

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The regularity of pattern of innervation in skeletal muscles of vertebrates, and especially in mammals, has been noted by many workers. Controversy existed at one period over the occurrence of multiple-innervated muscle fibres, Agduhr (1916, 1939) asserting that individual muscle-fibres in certain muscles of mammals possessed several nerve-endings, each innervated by a nerve-fibre from a different segment of the spinal cord. Multiple innervation has been demonstrated for the sartorius muscle of Anura, morphologically by Hines (1927) and physiologically by Katz and Kuffler (1941), but there is no reliable evidence of the existence of a similar condition in mammals (Wilkinson, 1929; Denny-Brown and Pennybacker, 1938).

Harrison (1910) suggested that the explanation of this regular pattern of innervation was to be found in changes of physiological state brought about in the tissues by the process of innervation; thus the formation of a nerve-ending on a muscle-fibre would so alter its physiological state as to prevent other nerve-fibres from innervating it.

Erlacher (1915) using the technique of direct implantation of peripheral nerves into denervated and normal muscles of monkeys and guinea-pigs, concluded that functional connection could be achieved by the implanted nerve with denervated muscle-fibres, and even with innervated muscle-fibres in normal muscles. He found that implants into normal muscles always gave isolated contractions when stimulated, and histological examination indicated that nerve-fibres from the implant had grown out and formed endings on normal muscle-fibres.

Steindler (1915, 1916) made an extensive study of nerves implanted into denervated muscles, reporting that rapid and effectual re-innervation was achieved. He made no systematic study of implants into normal muscles, but considered that it was unlikely that innervated muscle-fibres could be "hyperneurotized".

Elsberg (1917) studied the fate of nerve implants into denervated and normal muscles, stimulating the implanted nerves with an induction coil, and recording the contraction of the muscle mechanically. He asserted that, while implants into denervated muscles produced active contraction on stimulation, implants into normal muscles never did so. He also claimed that, if the normal nerve-supply of a muscle was severed and implanted into another part of that muscle together with a foreign nerve, the nerve which normally supplied the muscle readily re-innervated, while the foreign nerve failed to do so. From this he concluded that an affinity exists between a particular nerve and the tissue it normally innervates. Weiss and Hoag (1946) after conducting carefully controlled experiments, rejected this hypothesis.

Stookey (1924) utilizing direct implantation methods, obtained evidence suggesting that experimental hyperneurotisation was possible; stimulation of either the normal nerve-supply

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or the implanted nerve produced contraction of the operated muscles. No special precautions were taken to avoid injury to the innervation of the muscle when implanting foreign nerves, either by Stookey, or any of the other workers mentioned above.

Fort (1940) in a very extensive study of the problem of experimental hyperneurotisation, concluded that implants into normal muscles seldom led to functional connection, although he persistently observed localised contractions in the region of implantation when the nerve was stimulated. He concluded that only denervated muscle-fibres could be innervated from the implant, that outgrowth from the implant was more rapid in denervated than in normal muscles, and that re-innervation of denervated muscle-fibres occurred only in a restricted region —in or near the site of the degenerated end-plate. He extended the receptivity principle of Harrison, suggesting that innervated muscle-fibres were completely resistant to hyperneurotisation, but became receptive again several days after denervation. He likened the growth of the foreign nerve into denervated muscle to the downgrowth of nerve-fibres into developing muscles in the embryo, and regarded the changes in muscles following denervation as a reversion to the embryonic condition.

Aitken (1950) further studied the fate of implanted nerve-fibres in normal and denervated muscles. In an earlier study (1949) he had found that fibres of a nerve implanted into denervated muscles matured further than those of an implant into normal muscle. In denervated muscle he observed extensive outgrowth and ramification of nerve-fibres from the implant, with the formation of both typical and atypical nerve-endings. In normal muscles, although he frequently obtained localised contractions by stimulation of the implant, he could find only rare nerve-endings to account for them. He suggested, as did Fort, the possibility that unconnected nerve-fibres could stimulate muscle-fibres to contract.

The evidence from experimental embryological sources is more favourable to the occurrence of experimental hyperneurotisation. Rogers (1929, 1932) grafted spinal cords into the brachial region of developing Amblystoma larvae, and in some cases the host limb became innervated from the grafted cord, as well as from its own. Whilst no direct evidence was obtained by Rogers that individual muscle-fibres were innervated by fibres from both cords, studies of the contractions elicited by stimulation of each nerve-supply separately suggested that the host spinal cord innervated every muscle-fibre, and that, therefore, any innervation from the grafted cord must have resulted in hyperneurotisation. Weiss (1950) transplanted fragments of spinal cord into the tail fin of Amblystoma larvae, and found that substantial innervation of the dorsal musculature, from this implanted tissue, resulted. If a limb was implanted into the same furrow as the spinal cord, less nerve-fibres passed from the grafted cord into the host muscles, and more to the limb. Since the larvae were well developed, it may be assumed that the host muscle was fully innervated before operation, so that innervation from the implant must represent hyperneurotisation. Weiss' suggestions that new muscle-fibres, developed since the operation, were the only ones innervated from the implanted cord, or that a substance diffusing from the implant "weakened the resistance of the surrounding muscle-fibres to hyperneurotisation" do not seem very plausible. Detwiler's general conclusion (1933) from the experimental embryological evidence was that the problem remained open.

Recently, in a study of the fate of interrupted nerve-fibres regenerating into partially denervated muscles, I have obtained results (Hoffman, 1951) which make desirable a reconsideration of the problem of hyperneurotisation in general. Under conditions of partial denervation, the denervated muscle-fibres are rapidly re-innervated by branches sprouted from surviving nerve-fibres; these grow out and penetrate the endoneurial sheaths of degenerated nerve-fibres, passing down and reforming the old end-plates (Edds, 1950; Hoffman, 1950). When the interrupted fibres later regenerate down their old endoneurial sheaths they reach the point where these have been penetrated by the new collaterals,

pass down together with them and fuse with the arborisations of the reformed end-plates, thus providing bisegmentally-innervated end-plates.

The experimental investigation described below was designed to examine and analyse differences in the behaviour of nerve-implants in normal and denervated muscles, and to determine whether trophic changes in denervated muscle-fibres or degenerative changes in the intramuscular nerves are responsible for these differences.

Material and Methods.

As the experimental animal I have used the albino rat. In all cases the tibial nerve was implanted into the pelvic quarter of the gracilis anticus muscle. This muscle was found to be particularly suitable, for it consists of a flat sheet of parallel muscle-fibres, innervated by a branch of the obturator nerve, which enters about one quarter from the pelvic end and trifurcates, all branches running distally. Fig. 1 shows the innervation-pattern in an entire gracilis anticus muscle, consisting of the three main branches of the nerve. To the left of this can be seen several severed nerve-trunks (*ne*), constituting the nerve-supply of the posterior portion of gracilis, and several blood vessels (*bv*). On the few occasions where any branch was seen to run proximally, it was a very small bundle supplying a single spindle, as well as a single sensory fibre supplying a tendon end-organ at the proximal end of the muscle. Gold-stained material confirmed that no end-plates were present in the pelvic 25–30 p.c. of the muscle. This region is appreciably larger than the nerveless region in the anuran sartorius.

Under nembutal anaesthesia, and with aseptic precautions, the pelvic portion of gracilis anticus was exposed and dissected free of fascia, a small incision being then made in its surface. A further incision through the posterior portion of gracilis enabled the tibial nerve to be severed and dissected free. The free end of the nerve was stitched into the incision in the muscle by means of a very fine cutting-edged needle and a suture of sterilized human hair. This suture material was found to be very satisfactory, as it sets up very little tissue reaction, and is not absorbed. As long as 150 days after suture, the hair can still be identified, surrounded by about 20μ of fibrous tissue. Fig. 2 shows a muscle with the neuroma (*neu*) formed by the implanted nerve, sixty days after operation. The implant site may be observed to lie well away from the nerve-supply of the muscle, and bundles of fibres pass out in various directions from the neuroma.

Inevitably a certain amount of damage is caused to the muscle during operation, as Fort and Aitken have pointed out, since incising the muscle must result in transection of some of its fibres. However, it is probable that incision causes less widespread damage than blunt dissection.

After periods of from 10–130 days, possible re-innervation was examined by electrical stimulation; operated animals were anaesthetised with nembutal, the implanted nerve exposed and dissected proximally, severed a centimetre or more from the site of implantation, and laid on tissue-paper impregnated with paraffin wax, which served to prevent leakage of current to the muscle. The nerve was stimulated by means of a concentric bipolar electrode supplied from a constant-voltage square-wave stimulator, operating mostly at 10 volts, with a frequency of 2–10 per second. The contraction of the muscle was observed by eye, with the aid of a dissecting lens. Staining techniques used were:

(1) The Loewit gold method, which has been used for the detailed study of nerve-fibres and endings.

(2) A modification of the Willis (1945) silver method for sections, recently shown to me by Mr. Willis, and as yet unpublished. Sections were of Carnoy-fixed material, sectioned at 10–20μ, and counterstained with Mallory's trichrome or phosphotungstic-acid-haematoxylin stains, which provide excellent detail of the muscle-fibre structure, Schwann cells, and interstitial tissues.

(3) The Liang (1947) Feulgen technique for nerves, which I have used for staining whole normal and experimental muscles, with their implants *in situ*. From such preparations, the relation between the normal nerve-supply and implant fibres may be determined, and these latter may be followed to their terminations. In Figs. 1 and 2 typical low-power views of whole muscles may be seen, the neuroma formed by an implanted nerve being shown in the lower left corner of Fig. 2 (*neu*).

In an attempt to obtain some means of measuring the differences in behaviour between nerves implanted into differing environments, end-plates have been counted. The figures provided are very approximate, since it is impossible to ensure satisfactory staining throughout the muscle, and also to count the irregular structures formed in the disturbed region immediately surrounding the site of implantation. However, the differences found are of such magnitude as to imply significance in spite of these sources of error. In each group complete counts have been made of three or four muscles, and a mean determined. Owing to the difficulties of accurate counting described above, no detailed statistical analysis has been attempted.

DESCRIPTION OF PLATES.

Figs. 1, 3 and 4 show normal gracilis anticus muscle of the rat, at various magnifications. Figs. 2 and 7–10 show implants into normal muscles; Figs. 5, 6 and 11–17 represent implants into normal muscles close to denervated muscle masses; and Figs. 18–21 represent implants into denervated muscles.

PLATE 1.

Fig. 1. Entire normal gracilis anticus muscle of the rat, showing the distribution of nerve branches to the distal end (right). To the left of this nerve may be seen several blood vessels while further left are some severed nerve-stumps from the nerve-supply of the lower part of gracilis. The pelvic (left) quarter of the muscle is devoid of nerves. From a whole mount of a muscle stained by the Liang-Feulgen method, × 7.

Fig. 2. Gracilis anticus muscle with a 60-day implant in the pelvic, nerveless region. The neuroma (*neu*) is prominent, and bundles of nerve-fibres may be seen to emerge and travel in various directions. Liang-Feulgen, × 9.

Figs. 3 and 4. Normal gracilis anticus, showing normal nerve-fibres and motor end-plates. Note the regular character of the end-plates, and the prominent sole protoplasm and nuclei. Gold-stained, teased muscle, × 450.

Fig. 5. Two nerve-fibres emerging from the neuroma, pass amongst muscle-fibres, where one nerve-fibre forms a clumped ending in the lower centre of the field, whilst the other passes to the upper left of the field to form an ending, not quite in focus, which appears to be in the process of completion. From a 26-day implant into normal muscle close to denervated muscle. Gold, × 450.

PLATE 2.

Fig. 6. From a region some distance from the neuroma. A small bundle of nerve-fibres runs parallel with a group of regenerated muscle-fibres, and a small branch emerges to innervate the fibre to its right. The end-plate is clumped and raised into a "Doyere" eminence. From a 26-day implant into normal muscle close to denervated muscle. Gold, × 450.

Fig. 7. Well outside the neuroma, a small group of nerve-fibres runs alongside a group of muscle-fibres, one nerve-fibre innervating the muscle-fibre on its left, forming an end-plate which extends down the muscle-fibre. From a 54-day tibial nerve implant in normal muscle. Gold, × 450.

Fig. 8. In a region of intact muscle-fibres, a coarse, myelinated nerve-fibre runs alongside a muscle-fibre for some distance before forming on it a small atypical end-plate practically devoid of sole. From a 40-day implant into normal muscle. Gold, × 450.

Fig. 9. A more complex ramification of a nerve-fibre which has penetrated into intact muscle. The nerve-fibre, entering the plane of focus in the upper left of the field, forms a fairly large end-plate, from which fine fibrils pass out to form "en-grappe" extensions on the same muscle-fibre, to left and right. From a 44-day implant into normal muscle. Gold, × 450.

Fig. 1.

Fig. 2.

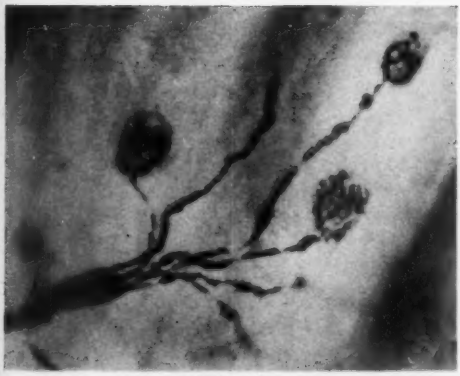

Fig. 3.

Fig. 4.

Fig. 5.

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Fig. 12.

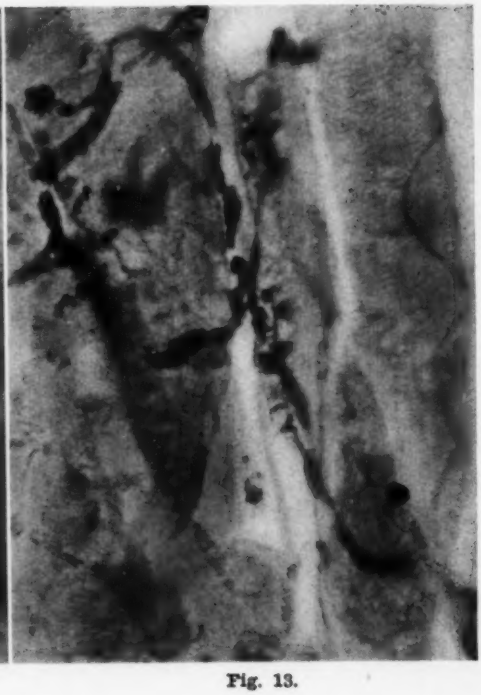


Fig. 13.

Fig. 14.

Fig. 15.

Fig. 16.

Fig. 17.

Fig. 18.

Fig. 19.

Fig. 20.

Fig. 21.

DESCRIPTION OF PLATES.

Plate 3.

Fig. 10. A 10μ section of the region immediately outside the neuroma formed after implantation into normal muscle. A thick, mature fibre cut across in the lower centre of the field, ramifies extensively, the branches passing upwards and twisting about the muscle-fibre which they accompany. These muscle-fibres are fine, and do not extend beyond the neuroma region; they are interpreted as fibres regenerated from fragments remaining after operation. All around them are bundles of nerve-fibres from the implant, randomly disposed. Willis silver method, × 350.

Fig. 11. In a region of intact muscle, fibres penetrating from the implant have formed a group of end-plates, including one unusually extended one in the upper part of the photograph. From an 84 day implant in normal muscle lying close to denervated muscle. Gold, × 450.

Fig. 12. Bundles of nerve-fibres pass up into intact muscle, where one fibre separates from the rest and forms an end-plate in the centre of the field. From an 84-day implant into normal muscle lying close to denervated muscle. Gold, × 450.

Fig. 13. From a region close to that portrayed in Fig. 11, showing the ramification of a group of nerve-fibres, and a number of end-plates formed by them. From an 84-day implant into normal muscle close to denervated muscle. Gold, × 450.

Plate 4.

Fig. 14. A bundle of nerve-fibres, passing into normal muscle, forms a number of nerve-endings. On the left, a large en-grappe ending is formed, and from it a further extension has grown upwards along the same muscle-fibre. In the upper right, several fine en-grappe endings are formed. From the same animal as Fig. 12. Gold, × 450.

Fig. 15. A bundle of nerve-fibres passes up from the neuroma into intact muscle, and from it a mature nerve-fibre passes to the right to form a very large and complex nerve-ending on a normal muscle-fibre. On the left other, smaller endings may be seen. From an 83-day implant into normal muscle, close to denervated muscle. Gold, × 450.

Fig. 16. Bundles of nerve-fibres, emerging from the neuroma, ramify amongst intact muscle. The nerve-fibres tend to align themselves parallel with muscle-fibres once they leave the larger bundles. Some fibres and bundles may be seen to turn back towards the neuroma. From a Liang-Feulgen stained whole mount of an 80-day implant into normal muscle close to denervated muscle. × 175.

Fig. 17. A low-power view of the emergence of bundles of nerve-fibres from the implant region, and their penetration into muscle (upper portion of field). The nerve-fibres pass up, between muscle-fibres, and form end-plates at various points. From an 84-day implant into normal muscle close to denervated muscle. Gold, × 150.

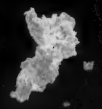
Plate 5.

Figs. 18 and 19. Thick, myelinated nerve-fibres, which have emerged from the neuroma, ramify throughout intact muscle, forming large numbers of endings. These endings are of varying character, but mostly en-grappe. The nerve-fibres entering the region are coarse and myelinated, but the branches which run into the end-plates are fine and unmyelinated. From a 150-day implant into denervated muscle. Gold, × 450.

Fig. 20. A single mature, myelinated nerve-fibre branches to form several fine, en-grappe endings on two muscle-fibres. From a 150-day implant into denervated muscle. Gold, × 450.

Fig. 21. A group of nerve-fibres, all mature and myelinated, enters the field on the lower right, forming a number of endings throughout the field. All the endings are fine and en-grappe. From the same animal as Fig. 19. Gold, × 450.

Various levels of magnification have been provided in order that reconstruction of the behaviour of implanted nerve-fibres may be facilitated. Fig. 2 provides a very low-power view (× 9) of the implant in relation to the surrounding muscle. Figs. 15 and 16 are of intermediate magnification, and illustrate the emergence of nerve-bundles, their orientation in regard to muscle-fibres, and their peripheral connections. The remaining figures provide high-power views of the detailed relations between nerve-fibres and muscle-fibres.



RESULTS.

Implants into normal muscles.

From 32–54 days after operation, animals with implants into normal muscles were anaesthetised and the implanted nerves severed and stimulated. In every case the muscle twitched, the region of the implant giving the greatest response. The frequency of stimulation was kept low enough to set up individual twitches, observable by eye. At frequencies above 8–10 per second the muscle summated to give a sustained tetanus. In no case did the magnitude of the contraction approach that elicited by stimulation of the intact obturator nerve.

Examination of the histological material showed that the implanted nerve formed a neuroma—a tangled mass of nerve-fibres running in all directions, within which lay a few fine muscle-fibres 6μ or even less in diameter, on some of which small end-plates, innervated from the neuroma, can be seen. Figs 5 and 6 illustrate this; compare these with the normal muscle-fibres and end-plates shown in Figs. 3 and 4. Outside the neuroma nerve-fibres mingle with groups of muscle-fibres, pursuing random courses amongst them, and forming end-plates on some. Fig. 10 is a photograph of a silver-stained section of this disorganised region, showing a bundle of fine regenerated muscle-fibres in amongst a tangled mass of nerve-fibres.

Surrounding this relatively disorganised region, probably corresponding to the area of the original incision, the muscle is intact and undisturbed, and is penetrated by nerve-fibres growing out from the neuroma. Most of the nerve-fibres run singly, passing between muscle-fibres, twisting around them and sometimes innervating. Fig. 7 is from such a region; a few nerve-fibres have penetrated between apparently normal muscle-fibres, and one nerve-fibre branches to form an ending on the muscle-fibre to the left of it. Many nerve-fibres turn back and run toward the neuroma; some accompany bloodvessels for some distance, then leave them to pass amongst muscle-fibres. The general pattern of orientation is conveyed by Fig. 16, a photograph of a region of a Feulgen-stained whole mount, although this shows more energetic growth than is found in implants into normal muscle. Figs. 8 and 9 illustrate the innervation of apparently normal muscle-fibres by implant nerve-fibres which have passed between them. Fibres grow both proximally and distally into intact muscle, forming nerve-endings as they proceed. Sometimes they travel long distances without branching, on other occasions they branch frequently as in Figs. 9 and 10.

The nerve-endings formed vary greatly in character, some being extremely simple, of grape-cluster (''en-grappe'' endings of Tschiriew) character, consisting merely of a few fibrillar branches, each ending in a swollen end-bulb. Others are large and complex, though usually ''en-grappe'' in character, and all intermediate forms may be seen. Ultraterminal endings are very common, and frequently several endings are formed on the same muscle-fibre. Secondary extensions from an end-plate out along the same muscle-fibre are frequently encountered, as in Fig. 9.

Nerve-fibres forming end-plates vary greatly in character; some are fine, non-myelinated and plexiform, as in Figs. 6 and 7, while others are coarse, myelinated and mature as in Fig. 8. The fibre forming the nerve-ending in Fig. 5 is intermediate in maturity. The great majority of nerve-fibres do not, however, form end-plates, but remain free in the perimysial spaces, tipped by growth cones, suggesting that they were still growing at the time of sacrifice. The silver-Mallory stained sections reveal that the growing nerve-fibres are thickly invested by hypertrophied Schwann-cells, which are reduced in numbers, however, when the fibres form end-organs and acquire endoneurial sheaths.

Implants into un-innervated (as distinct from denervated) muscles.

The short, fine muscle-fibres in the region of the neuroma have probably originated from muscle-fibres damaged during operation. Le Gros Clark (1946) has shown that interrupted muscle-fibres extend into connective tissue spaces, the newly-formed regions acquiring myofibrils and cross-striation, but their diameter remaining below normal for some time. Pogogeff

and Murray (1946) found that, in tissue culture, even small fragments of adult mammalian muscle would regenerate entire muscle-fibres. Thus these fine fibres within the neuroma probably originate from small fragments left after operation. Fig. 10 shows the appearance of these fibres, lying amongst masses of tangled nerve-fibres. The fine fibres seen in Fig. 6 probably represent regenerated regions of interrupted muscle-fibres.

The ability of nerve-fibres from the implant to innervate such regenerated regions of muscle-fibres, or entire newly-formed muscle-fibres, suggested the desirability of studying the growth of nerve-fibres into regions of muscles in which every fibre has been divided and is regenerating.

Experimental.

With fine flat-ground artery-forceps, the exposed gracilis anticus muscle was crushed just proximal to the point of entry of the nerve, thus separating the proximal 25–30 p.c. (nerveless) region from the innervated part by a crushed zone about 1 mm. wide. Longitudinal sections, stained with phosphotungstic-haematoxylin, confirmed that no muscle-fibres survived in this zone immediately after crushing, but that interrupted fibres on either side started to extend across it after about three days. Small fragments, left by the crush, could be seen regenerating new fine muscle-fibres.

(a) From material thus prepared, certain decisions can at once be made regarding the mechanism of re-innervation of denervated muscle-fibres, as is observed in partially denervated muscles. From the proximal (nerveless) zone, regenerating muscle-fibres extend across the crush and grow lengthwise between the muscle-fibres in the distal (innervated) zone, from which they are readily distinguished by their small diameter. Despite the close proximity of nerve-fibres and end-plates, these thin fibres do not reveal any sign of innervation. Yet, in normal muscle, the denervation of a proportion of the fibres results in prompt re-innervation from the surviving nerves (Edds, 1950; Hoffman, 1950). It seems to follow that absence of innervation is not, in itself, the source of the stimulus which causes sprouting (''neuroclesis'') of nerve-fibres, leading to re-innervation, but that the stimulus arises from the degenerating nervous tissue, as suggested earlier (Hoffman, 1950).

(b) In the next series of experiments, tibial nerves were implanted into the uninnervated muscles formed by crushing across the proximal region. Any differences in behaviour between implants into these and denervated muscles would differentiate the relative rôles of muscle-fibre changes and of nerve degeneration respectively, in stimulating nervous growth and terminal connection.

The animals were examined 26–43 days after operation. Stimulation of the implanted nerve produced a twitch of about the same magnitude as in implants into normal muscle, although localisation to the implant region was increased. In general the operated region of the muscle greatly increased in vascularity, and large numbers of fine regenerated muscle-fibres were encountered. The neuroma was essentially similar to that observed in implants into normal muscle, and enclosed small fine muscle-fibres, innervated from the neuroma. The extent of outgrowth of nerve-fibres from the neuroma was of the same order as observed previously, and axons passed amongst regenerated and normal muscle-fibres, innervating both. To some extent the scar formed in the crush zone limited the progress of invading axons, but nevertheless, some penetrated and innervated on the other side. These observations may be correlated with the increased, but still incomplete localisation of contraction to the vicinity of the implant.

The general picture of the innervation of muscle in this region was not appreciably different from that found in implants into normal muscle. Nerve-fibres pursued similar paths and innervated at random as before, normal and regenerated muscle-fibres being innervated indiscriminately. A rough quantitative estimate showed about 17 end-plates per muscle, both for the normal and ''crushed'' muscles. (Three specimens were studied in each series, and means of 17·7 obtained in each case.) Thus it appears that separation of a muscle-fibre-portion

from its innervated part does not affect the likelihood of its innervation by a foreign nerve-fibre.

In spite of the great variation in the appearance of the invading nerve-fibres, in general, it is possible to conclude that those which establish peripheral connections mature further than those which remain unconnected. This, in general, is in agreement with Aitken's (1949, 1950) observations. The endings formed on regenerated muscle-fibres are usually small and simplified, consisting merely of a few small branches under the sarcolemma (see Figs. 5 and 6). Very little sole sarcoplasm collects around these branches, few nuclei appear, and the sarcolemma is commonly raised to form a hillock, resembling the Doyere eminence seen in arthropod motor nerve-endings (see Fig. 6).

Fort and Aitken considered results, such as those described above as obtained from normal muscle implants, to be due to denervation of some muscle-fibres by accidental nerve-damage during operation, or to division of some of the muscle-fibres, thus providing denervated or uninnervated muscle-fibres which would be receptive to innervation. Both authors attempted to avoid nerve-damage, although the complex muscles used by Aitken probably do not have large enough portions devoid of nerve-fibres to ensure this. In the present study nerve-damage is clearly eliminated, while, although muscle-damage is inevitable when implanting the nerve, regenerating muscle-fibres may readily be distinguished, and their significance has been studied by implanting nerves into "crushed" muscles, in which every fibre is uninnervated and regenerating. The indiscriminate innervation of normal and regenerated fibres, and the innervation of muscle-fibres far from the site of injury indicate that actively growing nerve-fibres can hyperneurotize innervated muscle-fibres, as well as uninnervated and regenerated ones.

Implants into denervated muscles.

The branch of the obturator nerve supplying gracilis anticus was severed, dissected some distance proximally and covered with a methyl-methacrylate cap (Edds, 1945) to prevent regeneration of its fibres back into the muscle. The tibial nerve was then implanted as described above, and the animals examined 51 days later.

In all cases stimulation produced a much larger twitch than in normal or crushed muscles, although not as great as that produced by stimulation of the obturator nerve in normal muscles. The localised component of the contraction could still be observed in the vicinity of the implant.

Histological examination indicated a far more extensive outgrowth from the neuroma than in implants in normal or crushed muscles. Instead of individual fibres penetrating up amongst the intact muscle, whole bundles emerged, following similar paths to those observed previously; most growth was longitudinal once the region of disorganisation immediately around the neuroma had been passed. Fine, regenerated muscle-fibres could still be observed within the neuroma, innervated locally; these probably are responsible for the local component of the contraction. The ramification of the nerve-fibres within the muscle, and the endings formed, resembled those seen previously, but innervation was far more extensive. Entire bundles of innervated muscle-fibres were encountered, with complex sprays of fine "en-grappe" endings such as are shown in Figs. 18, 19, and 21. Fig. 20 illustrates the formation of a group of very fine endings by the branching of a single mature, myelinated nerve-fibre. The paths taken by the nerve-fibre bundles leaving the neuroma appear to be random; the pattern of innervation established is, in all cases, very different from that found in normal muscle (see Figs. 3 and 4). Multiple innervation of muscle-fibres may be observed, as in Figs. 18 and 19, together with complex relays of ultraterminals. Nerve-fibres may still be seen wandering in perimysial spaces, but whether they are sensory fibres unable to form connections, or motor fibres still capable of innervating, cannot be determined from their appearance.

In some cases, owing to defective capping, the severed nerve-supply of gracilis anticus managed to regenerate and grew back along its old pathways, restoring the normal pattern

of innervation in the muscle. All the endoneurial sheaths were entered, and all the old end-plates reconstituted, in spite of the extensive connection acquired by the implanted nerve. This further indicates that the processes of innervation, proximally by fibres from the implant, and distally by regenerating obturator fibres, proceeded independently.

To test this conclusion further, a series of experiments was carried out in which the obturator branch was crushed 100 days after tibial implantation into normal muscles. By this time the implant should have ramified extensively throughout the muscle, and should be capable of rapid response to any changes brought about by denervation, while the obturator innervation would be capable of rapid regeneration; thus, if competition between nerve-fibres for muscle-fibres is a reality, it might be expected to occur here. The animals were examined 22–43 days after operation, and in every case the entire muscle twitched on stimulation of the tibial nerve. Stimulation of the obturator nerve also produced a contraction, and in one case the contractions produced by the two nerves were of approximately equal magnitude. Histologically the obturator fibres appeared to have fully regenerated, filling all endoneurial sheaths, and reconstituting all end-plates. The fibres from the implant had achieved very extensive connection, resembling the growth of implants into permanently denervated muscles.

Throughout these experiments the only differences noted between implants into normal and denervated muscles were differences in rate and energy of growth and ramification. Fort considered that implanted nerve-fibres grew faster in denervated than in normal muscles, and considered alternative explanations: either movements and contractions of the normal muscles prevented nerve-fibres from establishing connection, or a secretion from the degenerated nerve or the denervated muscle-fibres stimulated growth of the implant fibres. To test the former alternative, he partially denervated muscles and found that implants into these acquired appreciable connection, even though they must have been buffeted by contractions of the muscle. Since, however, the re-innervation of partially denervated muscles by collateralisation from the surviving nerves is a rapid process (Hoffman, 1950) it is probable that it was complete before implant fibres had advanced into the muscle, so that these results further support the occurrence of experimental hyperneurotisation.

Counts of the number of end-plates formed by nerves implanted into denervated muscles resulted in a mean of 126·8 for four muscles. In spite of the difficulties and sources of error in these counts, when compared with the mean for normal and crushed muscles (17·7) this result is significant, and establishes the existence of some stimulus which accentuates the normally slight tendency of invading nerve-fibres to innervate muscle-fibres.

That it is a chemical substance, released from degenerating nerve-fibres which stimulates the surviving nerve-fibres to sprout new branches—in partially denervated muscles—has already been stressed (Hoffman, 1950); Koechlin and von Muralt (1947) have investigated a substance of low molecular weight, obtained by dialysis from homogenates of nervous tissue, which accelerates (up to four-fold) the regeneration of interrupted corneal nerves when injected intraperitoneally. Van Harreveld (1947) demonstrated the acceleration of recovery of partially denervated muscle, when overlying totally denervated muscle masses, and he attributed this to the diffusion of some substance from the totally denervated muscle into the partially denervated one, resulting in increased stimulation of the sprouting nerve-fibres. There seems to be some relation between, on the one hand this acceleration of recovery of partially denervated muscle, and on the other the increased growth of an implanted nerve in totally denervated muscle; the following experiments were accordingly designed to test the possible humoral basis for this latter phenomenon.

Implants into normal muscle lying close to denervated muscle.

In a series of animals the femoral nerve was divided, in some cases being capped to prevent its regeneration, concurrently with the implantation of the tibial nerve. The femoral nerve was approached by the method of Weiss and Edds (1945), enabling the nerve to be

severed and capped without endangering the (obturator) innervation of gracilis anticus. Animals were examined from 13–92 days after operation.

As early as 17 days after operation the muscles contracted on stimulation of the implant, and after longer periods substantial contractions were obtained, approaching those elicited by stimulation of the obturator innervation. The tendency to tetanise at low frequencies (8/sec) was again observed.

As early as 17 days after implantation, nerve-fibres could be seen emerging from the neuroma, and invading the surrounding muscle. After 22 days, the penetration was extensive, and sprays of "en-grappe" nerve-endings could be seen, many connected with mature nerve-fibres. After longer periods, bundles of nerve-fibres could be observed, running into the muscle and forming sprays of nerve-endings as they advanced. In Figs. 16 and 17 low power views are presented of the invasion of the muscle by implant fibres, and (in Fig. 17) the formation of nerve-endings. Figs. 11–15 illustrate the formation of widely varying types of nerve-endings in intact, innervated muscle by the invading nerve-fibre bundles. In some regions almost every muscle-fibre was innervated by implant fibres, and in some cases fibres were multiply-innervated at intervals by a parallel-running nerve-fibre (as in Fig. 11).

The mean of the counts of nerve-endings in three muscles was, in this case, 91·3; by comparison with means for normal and crushed muscles on the one hand, and denervated muscle on the other, it may be seen that the stimulus acting on the implant fibres produces far more innervation, and approximates to the conditions encountered with implants into denervated muscles.

Whilst not completely eliminating other possibilities, these experiments seem to support the view that it is a chemical substance which stimulates nerve-fibres implanted into denervated muscles to innervate vigorously, and these results are essentially in agreement with the findings of van Harreveld.

DISCUSSION.

The observation that newly-formed regions of muscle-fibres, and even fibres regenerated from small fragments in the neuroma region, can be innervated by nerve-fibres from the implant is of interest, since it indicates a great lability of the neuromuscular system, even in the adult mammal. It is in agreement with the findings of May (1949) who transplanted fragments of newborn mouse brain and muscle to the anterior chamber of the adult mouse eye, and found that nerve-fibres grew out and formed simple endings on the muscle-fibres. When considered together with the occurrence of nerve-endings in the normally nerveless pelvic portion of gracilis anticus, following implantation, Fort's suggestion that re-innervation is only possible in a restricted region of the muscle-fibre—at or near the degenerated end-plate—does not find support.

The type of nerve-ending formed seems to be determined partly by the calibre of the nerve-fibre forming it, and partly by the region of the muscle-fibre innervated. Fine nerve-fibres form only diminutive endings, while thick ones may form large complex endings, or wide-spread "en-grappe" types. Maturation following peripheral connection must be taken into account, but since the long periods allowed have provided the opportunity for maturation, the failure of connected fibres to mature must be attributed to other factors. Indications have been obtained previously (Hoffman, 1951) that if a nerve-fibre forms many extra branches, it becomes overloaded, and maturation of these new branches

is delayed. It must also be remembered that some of the connected implant fibres form fewer branches than they would normally. Endings on fine, regenerated muscle-fibres are simple and cramped, contrasting with the expanded endings seen on full-sized muscle-fibres. This suggests that the space available under the sarcoplasm is a factor in determining the spread of the hypolemmal axon tip.

The sole-plate formed at abnormal sites by implant-innervated end-plates contrasts with that of the normal end-plates. The axon branches do not seem capable of collecting much sarcoplasm, or many nuclei, and the sarcolemma is often raised in a "Doyere" eminence. This suggests that in the mature muscle-fibre the sole-plate is less readily organised by the branching nerve-fibre, possibly due to the sparcity of granular protoplasm. This difficulty is not encountered when old end-organs are re-innervated, regenerating a typical end-plate with ample sole (Gutmann and Young, 1944; Hoffman, 1950).

The frequent occurrence of contraction-summation at very low stimulus frequency recalls comparable observations of Thompson, Morgan and Hines (1950) in regard to recently re-innervated muscles. Since, in the observations recorded above, the muscle-fibres were (in some cases) normal, and in fact innervated normally at other points, it seems probable that this peculiarity is associated with atypical end-plates, and is probably an attribute of the fine branches and small, simple end-bulbs associated with "en-grappe" endings and immature "en-plaque" forms.

The physiological observations recorded above merit some consideration in their relation to the observations of previous workers. Erlacher, working with complex muscles, observed local twitches following stimulation of implants into normal muscles, and more powerful—but still local—contractions if the muscle had previously been denervated. Fort, utilising a parallel-fibred muscle (toad sartorius) observed small local contractions in implants into normal muscles, while a more powerful and generalised contraction followed stimulation of an implant into denervated muscle. Aitken, like Erlacher, observed only local twitches, whether the muscle was innervated or denervated, although the latter provided larger contractions when the implant was stimulated. Since both Aitken and Erlacher utilised complex muscles, in which individual fibres are short and arranged around a central tendon, innervation of a restricted region of the muscle by nerve-fibres escaping from the neuroma would result, on stimulation of the implant, in contraction of a group of short muscle-fibres, thus causing a squirming or local contraction of the muscle. As regards the results recorded by Fort, the local contraction obtained is probably comparable to that obtained in the experiments described earlier in this paper, and may be attributed to the contraction of muscle-fibres regenerated within the neuroma which, since they are short, resulted in a similar localised contraction. Fort's suggestion that unconnected nerve-fibres can stimulate contraction of muscle-fibres by the

release within the perimysial spaces of acetyl-choline was based on his failure to find nerve-endings to account for his local contractions. Since such endings have been regularly detected in the experiments described above, the hypothesis seems unnecessary.

The essential difference between the growth of implants into normal and denervated muscles lies in the vigour of growth, ramification and peripheral connection. Muscle-fibres either innervated or deprived—by interruption—of their connection with innervated regions are innervated by implanted nerve-fibres in a random fashion to approximately the same extent. Muscle-fibres denervated by nerve-section, or innervated but lying close to denervated muscle-masses, are innervated much more energetically, but in the same random fashion. The presence of a normal nerve-ending in no way affects the formation of another ending on the same muscle-fibre by a nerve-fibre from an implant, nor does the presence of these latter endings affect the regeneration of the normal innervation of the muscle.

The possibility that endings could be formed on an innervated muscle-fibre some distance from the first end-plate, but not close to it, might explain some of the discrepancies in the literature; this is rendered less likely, however, by the observation (Hoffman, 1951) of bisegmental experimental hyperneurotization at the original end-plate. Thus it appears that no mechanism of resistance to hyperneurotization actually exists.

The humoral agent apparently responsible for the stimulation of growth of nerve implants in denervated muscle is probably released from the degenerating nerve, since uninnervated muscle (as obtained by transverse crush) does not appear similarly to stimulate growth. If this agent is related to neurocletin (Hoffman, 1950), N.R. substance (Koechlin and von Muralt, 1947) or van Harreveld's accelerator (1947) it would appear likely that it is first released by the breakdown of the myelin following Wallerian degeneration. Since, however, the period over which the stimulus acts is much longer than in the case of sprouting produced by partial denervation, it is possible that the proliferated sheath cells ("cords of Büngner") may continue to secrete it, as suggested by Cajal and co-workers (1928) for their "neurotropic" substances. The secretion by sheath cells of a substance contained in myelin is rendered feasible by Speidel's (1933) assertion that these cells secrete myelin on the nerve-fibres of the developing tadpole's caudal skin.

The appearance of fascicles of nerve-fibres invading muscles after denervation, whilst only individual fibres penetrate normal muscle, may be a manifestation of this growth stimulation. Weiss maintains (e.g. 1950) that fasciculation occurs because pioneering fibres have made functional connection, following which an alteration in their physical surface-state causes other fibres to adhere to them and follow similar courses; however Detwiler (1928) and Hoadley (1925) have demonstrated that spinal nerves will grow towards abnormal

organs, such as brachial nerves towards a nasal placode, forming fascicles even if they do not innervate.

Recent experiments involving the grafting of mammalian tumours on to chick embryos (Levi-Montalcini, 1951; Levi-Montalcini and Hamburger, 1951) have resulted in extensive invasion of these grafts by autonomic and sensory nerve-fibres, although few tumour cells were actually innervated. Nerve-fibres in other parts of the chick were activated to invade abnormal organs (e.g. mesonephros on the seventh day), this effect resulting even if the graft was deposited on the chorio-allantois, some distance from the embryo. Ganglion cells showed the characteristic hypertrophy and hyperplasia which has previously been observed to follow enlargement of the peripheral field, and the authors attribute these phenomena to the release from the graft tumour of growth-stimulating substances. They further conclude that the establishment of peripheral connections is not essential for the characteristic peripheral influence on the development of the central nervous system to manifest itself.

The conception of Fort that the behaviour of nerve-fibres implanted into muscles can be sharply separated into stages of outgrowth into the muscle, followed only later by innervation of muscle-fibres, finds no support in the experiments described above. The process of outgrowth and innervation is continuous and random mechanical factors apparently determining where the fibres wander, and innervation may occur anywhere along the path of outgrowth. Similarly the conception of innervated muscle-fibres resisting hyperneurotization, and becoming receptive to innervation only some days after denervation, is contrary to the observations described above.

Since the receptivity theory of Harrison and Fort is apparently invalid, the suggestion that local peripheral factors regulate the pattern of development of muscle innervation (Weiss, 1950) must be discarded; however, experimental embryology has provided evidence in support of a possible mechanism of regulation of the development of peripheral innervation. Hamburger (1939) has demonstrated in chick embryos that the size of an organ or cutaneous region to be innervated regulates the number of neurones differentiating (Hamburger and Keefe, 1944) in the appropriate segments of the spinal cord. Extirpation of limb-buds causes hypoplasia and atrophy; while grafting supernumerary limb-buds causes hyperplasia and hypertrophy. Thomas and Davenport (1949) have demonstrated in adult rats, that the grafting of a small nerve to a larger peripheral nerve-stump results in increased branching of the regenerating fibres, satisfactorily innervating an increased peripheral field. Thus the number of nerve-fibres entering a muscle in ontogenesis is regulated centripetally by the number of muscle-fibres differentiating, while in regeneration along a nerve-trunk the endoneurial pathways exercise a similar regulative function. When further nerve-fibres are diverted into innervated tissue, hyperneurotization occurs, and the normal mechanism of regulation ceases to operate. On occasion,

even artificially evoked branches of normal, connected nerve-fibres may hyperneurotize (Hoffman, 1950).

It must be emphasized, however, that the observed occurrence of only a single end-plate on each muscle-fibre in mammals has yet to be fully explained.

SUMMARY.

Nerve-fibres, implanted into normal muscles, or muscle-regions functionally deprived of innervation by separation from their innervated regions, innervate them to some small extent. Nerve-fibres implanted into denervated muscles grow more actively and innervate more energetically. A similar degree of activity is demonstrated by nerve-fibres implanted into normal muscles lying close to denervated muscles. This suggests a humoral mechanism for the difference in activity.

Regenerated muscle-fibres and regenerated regions of interrupted muscle-fibres are innervated from the implant. The inhibition of hyperneurotization by the presence of a nerve-ending could not be demonstrated—in fact hyperneurotization occurred readily.

The normal pattern of innervation is apparently regulated by central factors in ontogenesis, and can readily be disturbed by peripheral alterations in the adult.

On the whole, the random courses pursued by nerve-fibres, and the irregularity of innervation, render direct implantation methods less satisfactory in re-innervation than the use of the old endoneurial pathways in the nerve-stumps.

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COMPARATIVE EFFECTS OF THE NICOTINE ALKALOIDS UPON RESPIRATION

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Hicks *et al.* (1947) described the effects of the nicotine alkaloids upon respiration, in experiments using Head's diaphragmatic slip to assess the respiratory activity. These results suggested that the laevo-alkaloids stimulate the respiratory centre whilst the dextro varieties have mainly a depressant effect. Since the diaphragmatic slip shows only one aspect of the total respiratory activity and may not be a true index of the ventilation, it seemed desirable to re-investigate the respiratory responses to the alkaloids using techniques which would simultaneously indicate the respective parts played by the thoracic, abdominal and diaphragmatic respiratory components in tidal air and minute ventilation.

EXPERIMENTAL.

Methods.

Rabbits (paraldehyde anaesthesia 1·7–1·8 ml./kg. body wt.) were used. The vagi were sectioned and a tracheal cannula inserted. Respiratory movements and minute volumes were recorded by methods previously described (Kerr, 1950). Subsequent procedures have been indicated in the text. The drugs, made up in Ringer or normal saline, were injected by cannula into the external jugular vein and washed in by saline.

Carotid body chemoreceptor response.

With doses high enough to stimulate the chemoreceptors, all the alkaloids exhibit the typical nicotine response of hyperpnoea and secondary depression. The depression is greatest after d-nor-nicotine. In some rabbits the hyperpnoea was preceded by a brief apnoeic period. The behaviour of the alkaloids toward the chemoreceptors is very similar to that described for the cervical ganglion by Hicks *et al.* (1947): l-nicotine stimulates at the lowest concentration, then d-nor-nicotine, whilst d-nicotine and l-nor-nicotine stimulate at much the same dose. The stimulating dose of d-nor-nicotine is very close to the toxic dose, so that respiratory failure often follows a hyperpnoea with this isomer. The actual dose range varies from rabbit to rabbit; Protocol 1 is an example.

Nicotine responses with inactivated chemoreceptors.

If the chemoreceptors are blocked by infiltration of the sinus region with isotone or cocaine, (or if the doses are so adjusted that hyperpnoea does not occur on injection of the alkaloid) a differential effect between the thoracic and abdominal records can be seen.

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With the laevo varieties the thoracic record is depressed in amplitude and often shows a base line shift in the expiratory sense whilst the abdominal record shows a base line shift in the inspiratory sense. (Fig. 1, A and C).

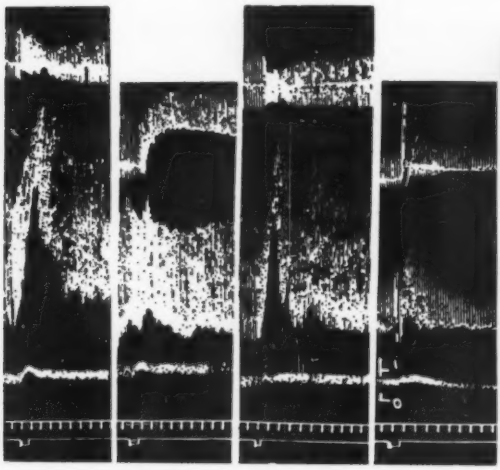

Fig. 1. Comparative respiratory responses to the nicotines in the absence of chemoreceptor responses. Rabbit 1,650 gm. Paraldehyde anaesthesia.

A. 0·2 mg. l-nicotine.
B. 1·0 mg. d-nicotine.
C. 1·0 mg. l-nor-nicotine.
D. 1·0 mg. d-nor-nicotine.

In this and subsequent figures the records from top downwards are respectively thoracic and abdominal respiration (inspiration upstroke), ventilation rate (read as mean of excursion, calibrated in litres/min.). Time marker in 6 secs. intervals. Signal line depression indicates moment of injection of alkaloid.

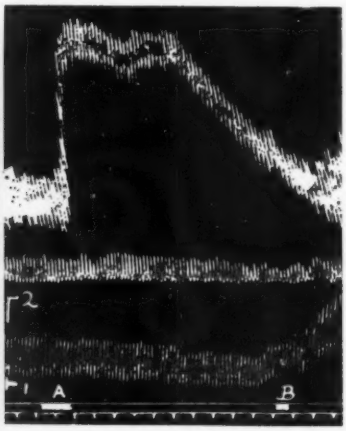

Fig. 2. Marked d-nicotine response. Rabbit 1,900 gm. Paraldehyde anaesthesia. At A 2 mg. d-nicotine injected, the ventilation rate becomes slightly depressed until switching the ventilation meter to the 10 p.c. CO_2 intake at B causes an abrupt shift in the record followed by a rise in ventilation as hyperpnoea develops.

With d-nicotine the response pattern is quite different, the thoracic record shows a sustained base line shift in the inspiratory sense with the abdominal record showing little change (Fig. 1, B.). This effect has not been seen to occur consistently with d-nor-nicotine (Fig. 1, D.). When the sinus mechanism is still intact there is little evidence that d-nor-nicotine with stronger doses does more than produce a brief hyperpnoea. The results illustrated in Fig. 1 were able to be repeated on the one animal with varying order of injection of the isomers, as can be seen also from Protocol 1, a different experiment from that in Fig. 1. Some caution in interpreting the thoracic amplitude changes is necessary as has been shown in an earlier paper, (Kerr, 1950 Fig. 2), and it seems safer to refer to the minute ventilation in deciding if a dose of nicotine alkaloid is "stimulating" or "depressing". Ultimately continued injection of all the alkaloids lowers the ventilation rate. The differential "tonus" responses produced

by the isomers in this series may be either of central or peripheral (nicotine contracture) origin. An attempt to analyse the toxic effects of the drugs upon the pattern of individual respiratory movements, as has been done with vagotomy in this previous paper, did not reveal any consistent differences between the alkaloids. It was noticed in some preparations that where thoracic respiration first failed this terminally resumed with gasping, as the diaphragm became inactive (Fig. 3). Fig. 2 has been included to show the most marked d-response encountered.

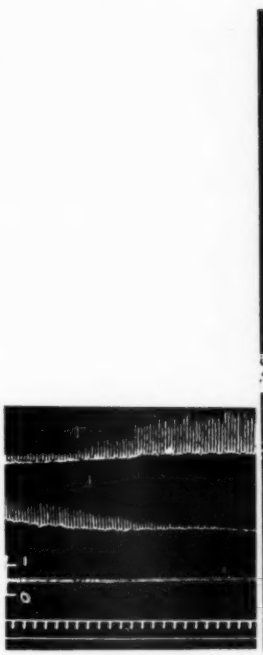

Fig. 3.

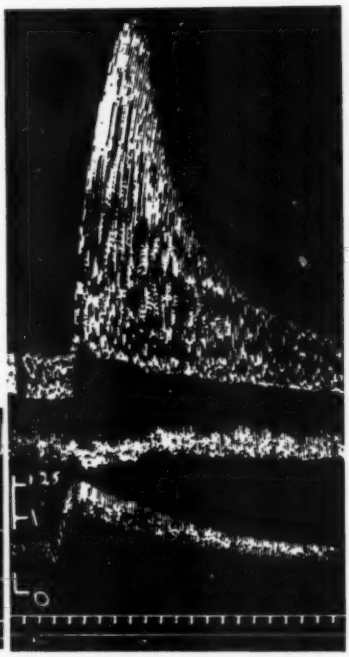

Fig. 4.

Fig. 3. Rabbit 1,600 gm. Paraldehyde anaesthesia. Failure of diaphragmatic respiration with compensatory increase in thoracic respiration maintaining the ventilation after 1·0 mg. of d-nor-nicotine. The animal died 24 seconds later after a period of gasping confined to the thorax.

Fig. 4. Hyperpnoea in the absence of diaphragmatic respiration. Rabbit 1,520 gm. Paraldehyde anaesthesia. Injection of 5 mg. l-nicotine. This hyperpnoea follows 8 minutes after a previous injection of 5 mg. l-nicotine which produced a hyperpnoea of twice the intensity and duration of that shown here.

Hyperpnoea in the absence of diaphragmatic respiration.

Larger doses of l-nicotine, with the chemoreceptors blocked and the phrenics crushed near their origin, produce a prolonged hyperpnoea with a sustained shift of the base line in the inspiratory sense. Lower doses of l-nicotine do not produce this inspiratory shift (Fig. 4). It is not possible to say if this is through the same mechanism by which d-nicotine alters the

thoracic base line. The hyperpnoea produced under these circumstances is smoother and more prolonged than the "explosive" hyperpnoea, generally accompanied by somatic movement, which follows on stimulation of the sino-aortic chemoreceptors with nicotine. The latter hyperpnoea is often more apparent than real, the movements being ineffectual for ventilation with the period of increased ventilation lasting only a few breaths.

DISCUSSION.

The present experiments offer a further example of the important pharmacological differences between the dextro and laevo alkaloids of the nicotine group. It is confirmed that d-nor-nicotine is more toxic than the other three alkaloids (Hicks and Sinclair, 1947).

So far as respiration is concerned the chemoreceptors are stimulated by intravenous doses of the alkaloids which group themselves much as do the doses stimulating the cervical ganglion; this offers some support to the concept that nicotine stimulates the chemoreceptors in a ganglionic synapse rather than at the pH-sensitive bodies themselves (von Euler *et al.*, 1939).

The stimulating dose of d-nor-nicotine approximates to the toxic dose in the rabbit, considerably higher concentrations of the other alkaloids being tolerated. Respiratory depression and failure relate more to the curariform action of the alkaloids on the respiratory musculature than to a central toxic action (Gold and Modell, 1936). It was concluded by Hicks *et al.* (1935) that d-nor-nicotine is more potent in reversibly blocking indirect stimulation of the frog gastrocnemius preparation than is l-nicotine. If these results can be referred to warm blooded musculature they offer some explanation of the fact that d-nor-nicotine depresses respiration more rapidly than do the other alkaloids, although repeated injection of all four drugs gradually depresses the ventilation. We were unable, on the basis of ventilation rate, to confirm the general conclusions of Hicks *et al.* (1947) so far as concerns the grouping of respiratory responses into depressant dextro- and stimulant laevo-alkaloids. However, we used a different method for assessing the respiration and it is of interest that in the animals where the chemoreceptors were inactive the respiratory patterns did tend to be grouped into responses according to optical activity. There seems to be no ready explanation of the precise relationships between the earlier results with the diaphragmatic slip and the present results where both thoracic and abdominal respiration were qualitatively recorded.

The behaviour of the muscle groups toward toxic doses of the alkaloids in the present experiments seems to provide some further evidence that there is a degree of independence of central control over thoracic and diaphragmatic respiration (as suggested previously, Kerr, 1950) where first one muscle group then the other attempts to compensate for failure to maintain adequate ventilation. There is the possibility that these results are to be explained entirely in terms of peripheral drug actions.

The prolonged hyperpnoea in the rabbit surviving on thoracic respiration may be due to a direct action of the nicotine on the respiratory centre. In some ways it resembles the hyperpnoea due to peripheral afferent stimulation. Apart from the sino-aortic chemoreceptors, various other receptor sites have been suggested (Schmidt and Comroe, 1940) and in this regard Brown and Gray (1948) have shown that close arterial injection of nicotine promotes discharge from sensory endings in skin and mesentery. Some such afferent source may be responsible in part for the prolonged hyperpnoea observed.

SUMMARY.

The respiratory responses to the nicotine alkaloids have been re-examined using rabbits (vagotomised and under paraldehyde anaesthesia).

The sino-aortic chemoreceptors produce hyperpnoea with the alkaloids in the following dose order: l-nicotine (in lowest concentration) d-nor-nicotine, then d-nicotine and l-nor-nicotine (in similar, higher concentration). The stimulating dose of d-nor-nicotine approximates to the toxic dose, considerably higher concentrations of the other alkaloids being tolerated. The dose range varies from rabbit to rabbit.

Differential patterns of response of thoracic and abdominal musculature can be observed, particularly in the absence of the chemoreceptor response.

The laevo-alkaloids tend to shift the thoracic record base line towards expiration, and in the abdominal record towards inspiration.

D-nicotine produces a sustained thoracic record base line shift towards inspiration, the abdominal record showing little change.

D-nor-nicotine does not consistently produce this effect, its depressant actions apparently overriding the stimulant.

In the rabbit surviving on thoracic respiration, after phrenotomy and with the sino-aortic chemoreceptors inactivated, the injection of large doses of l-nicotine produces a prolonged hyperpnoea differing in character from the sino-aortic response.

Previous conclusions that the dextro alkaloids are depressant and the laevo stimulant toward the respiratory centre have not been confirmed.

Acknowledgment. We are indebted to Prof. Sir C. Stanton Hicks who kindly supplied the alkaloids for this work.

PROTOCOL 1.

Rabbit. 2,200 gm. Female. Paraldehyde anaesthesia.
A.T. = Abdominal movement as recorded by the method.
T.T. = Thoracic movement as recorded by the method.
V.R. = Ventilation rate, litres/min. inspired air through Gaddum meter.

Time	Injected dose	Result, tracing summary
14·48	0·1 mg. l-nic.	A.T. base line shifts slightly in inspiratory sense, amplitude increases briefly. T.T. base line shifts markedly in expiratory sense without change in amplitude. V.R. 1 l./min. increases by 10 p.c. for ½ minute.
14·55	1 mg. d-nic.	No respiratory response. V.R. 1 l./min.
15·00	0·15 mg. l-nic.	Brief hyperpnoea, somatic movement. A.T. marked base line shift in inspiratory sense. T.T. decreased amplitude. V.R. 1·25 l./min. briefly 2·25 l./min. declining to 1·0 l./min. after hyperpnoea.
15·07	2·0 mg. d-nic.	Brief hyperpnoea, somatic movement. A.T. slight base line shift in expiratory sense, amplitude unchanged. T.T. marked base line shift in inspiratory sense, amplitude increased. V.R. 1·25 l./min. rising to 2·5 l./min. for 3 secs., declining to 1·5 l./min.
15·15	1 mg. l-nor-nic.	A.T. slight shift of base line in inspiratory sense Amplitude increased. T.T. Moderate shift of base line in expiratory sense, amplitude decreased. V.R. steady at 1·25 l./min.
15·20	0·125 mg. l-nic.	A.T. slight shift of base line in inspiratory sense, no amplitude change. T.T. base line shifts in expiratory sense for two minutes. V.R. steady at 1·25 l./min.
15·25	2 mg. l-nor-nic.	Brief hyperpnoea. A.T. marked shift of base line in inspiratory sense. T.T. Amplitude depressed base line disturbed by somatic movement during hyperpnoea. V.R. 1·25 l./min. rising to 2 l./min. for 3 secs., declining to 1·0 l./min. after hyperpnoea.
15·37	0·5 mg. d-nor-nic.	Brief hyperpnoea. A.T. slight shift of base line in inspiratory sense, amplitude increased. T.T. brief shift of base line in inspiratory sense, disturbed by somatic movement. V.R. 1 l./min. rising to 2·0 l./min. declining to 0·75 l./min.
15·40	1·5 mg. l-nic.	A.T. Marked shift of base line in inspiratory sense, amplitude unchanged. T.T. base line shift in expiratory sense, amplitude decreased three secs. after onset of these changes hyperpnoea declined as convulsions began. V.R. 1·25 l./min. rose to 2 l./min. then fell to 0·5 l./min. at onset of convulsions. Expt. terminated.

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